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The Mechanisms of Autohaemotherapy with Heat, Oxygen/Ozone and Ultraviolet Treated Blood in Vascular Diseases

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
APAAP	alkaline phosphatase/anti-alkaline phosphatase
BH4	tetrahydrobiopterin
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
CIM	cell - mediated immune
CSFs	colony Stimulating Factors
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDRF	endothelium-derived relaxing factor
ELISA	enzyme-linked immunosorbent assay
fmol	femtomolar (10^{-15} mol)
HLA	human leukocyte antigen (human histocompatibility complex)
HOU	heat, ozone/oxygen and ultraviolet
HSP	heat shock protein
ICAM	intracellular adhesion molecule
IFNs	interferons
ILs	interleukins
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NADPH	reducted nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor-kB
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PECAM	platelet-endothelial cellular adhesion molecule
pg	pilogram (10^{-12} g)
PG	prostaglandin
PGI_2	prostacyclin
PLT	platelet
PLT [c]	washed platelet from control untreated blood
PLT [t]	washed platelet from HOU-treated blood
PPP	platelet poor plasma
PPP [c]	platelet poor plasma from control untreated blood
PPP [t]	platelet poor plasma from HOU-treated blood
PRP	platelet rich plasma
PVD	peripheral vascular disease
RIA	radioimmunoassay
RNA	ribonucleic acid
RNI/RNS	reactive nitrogen intermediates/species
ROS/ROI	reactive oxygen species/intermediaters
Sd	standard deviation
TGF	transforming growth factor
TNF	tumour necrosis factor
TX	thromboxane
UV	ultraviolet
VAM	vascular adhesion molecule
μg	microgram (10^{-6} g)
μl	microlitre (10^{-6} L)
μM	micromolar (10^{-6} Mol)

ABSTRACT

In the last three decades, autohaemotherapy with blood treated with oxygen containing low concentrations of ozone, either alone or in combination with an elevated temperature and ultraviolet irradiation (HOU), has been used in Europe for the treatment of peripheral vascular diseases. Many apparently widely differing beneficial claims have been made for this therapeutic approach. This study has provided an objective rationale as to how HOU-treated blood administered as autohaemotherapy may work as a therapeutic agent.

This study has investigated the effect of the treatment of blood *in vitro* with HOU on the blood platelets. The results indicate that either in healthy volunteers or in patients with PVD and/or diabetes, HOU-treated blood causes a dose-dependent, reversible inhibition of blood platelet aggregation in response to ADP and collagen, calcium ionophore A23187 and thrombin. The treatment of whole blood with HOU did not result in the destruction of platelets, as indicated by a lack of increase in release of the alpha-granule component platelet-derived growth factor into the plasma, and a increase in total platelet count. The concentrations of endothelium-derived relaxing factor (EDRF, known to be closely related to or identical with nitric oxide) and prostacyclin (PGI_2) released from immune cells in the HOU-treated blood are increased by this treatment *in vitro*. EDRF and PGI_2 are both well known inhibitors of platelets aggregation and vasodilators. Evidence that the observed inhibition of platelet aggregation in treated blood is at least in part caused by EDRF production is provided by the reversal of the inhibition in the presence of oxy-haemoglobin, an EDRF inhibitor.

An *In vivo* clinical study was performed on normal healthy volunteers using autohaemotherapy with blood exposed to UV light and ozone in medical oxygen (15 μg/mL) at a temperature of 42.5 °C. These volunteers were monitored by measurement of standard haematological parameters, a clinical chemistry profile and clinical symptoms and signs. These was no evidence of harmful effects of the treatment.

In vivo HOU-autohaemotherapy enhanced the expression of the activation markers IL-2R, Ber-Mac3 and HLA-DR on peripheral blood mononuclear cells demonstrating for the first time objectively -measurable systemic changes in treated individuals. There was also significant increase in plasma prostacyclin concentrations measured as its stable metabolite 6-keto-$PGF_{1\alpha}$ after HOU-autohaemotherapy.

Endothelial dysfunction, with a reduction in the synthesis of vasodilators, particularly nitric oxide and perhaps also prostacyclin, plays a critical role in many vascular diseases. If autohaemotherapy with HOU-treated blood can restore endothelial function, as suggested by the evidence of an increase in prostacyclin levels in treated individuals, then this therapy could represent a major advance in the treatment of a number of vascular diseases.

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CHAPTER 1

Introduction

1.1 Background

Peripheral vascular disease (PVD) is an indication of generalized arterial disease. It is responsible for considerable morbidity in the general population, particularly amongst the elderly. Its prevalence increases with age and is higher in males than females (Fowkes, 1988). In one study, carried out in Edinburgh, almost one-third of middle-aged men were found to have significant peripheral arterial disease, and approximately 5% experienced symptoms of intermittent claudication (Fowkes, et al, 1991). The risks associated with lower-limb atherosclerosis are not primarily due to the disease itself, but to concomitant ischaemic heart disease and cerebrovascular disease, with almost half of the deaths among this group being caused by heart disease (Jelnes, et al, 1986). Indeed, the distribution of coronary and cerebrovascular disease throughout the population is similar to that of peripheral arterial disease (Fowkes, 1988) and represents an important indicator of generalized atherosclerosis. Atherosclerosis affecting the abdominal aorta is common and potentially lethal. Two to three percent of men over 50 have have an aneurysm of the abdominal aorta (Greenhalgh, 1990).

Occlusive vascular disease, which includes ischaemic heart disease and cerebrovascular disease is the single largest cause of death in the UK. In 1991 alone nearly 262,000 deaths (47.7 % of the total number) were attributed to diseases of the circulatory system (Office of population censuses & Surveys, 1991). In the UK. rupture or complications of abdominal aorta aneurysms kill 10,000 people each year (Greenhalgh, 1990). In the United States of America coronary heart disease alone accounts for over half a million deaths annually (Munro & Cotran, 1988).

Atherosclerosis affecting the lower limbs may require arterial by-pass surgery and, in serious cases, amputation and long-term rehabilitation. A study in the UK has found that arterial reconstruction costs around £7,750 per person (1989 prices) and amputation around £11,000 per person (Drummond & Davies, 1994). Aortic aneurysm is a major cause of death and the only effective treatment is sophisticated surgical operation, which is equally costly. Death is mostly the result of complications following amputation and reconstructive surgery. Surgery is justified only if claudication is severe and intractable and the patient must not have other diseases that will increase the risk and limit the benefits of operation - for example, angina or osteoarthritis.

1.2 Aetiology of peripheral arterial disease

As peripheral arterial disease and ischaemic heart disease are clinical manifestations of basically the same pathological process, it is not surprising that the risk factors for the two conditions are similar, particularly elevated levels of cholesterol (especially low-density lipoprotein LDL) and hypertension (Leng 1995).

Smoking, however, seems particularly important in the aetiology of lower-limb disease (Fowkes, 1989). Smoking increases the risk of peripheral arterial disease by up to seven-fold (Heliovaara, et al, 1978; Hughson, et al, 1978) and at least 75% of those presenting with intermittent claudication will be smokers (Fowkes, et al, 1991). Smoking may also be responsible for abnormalities in haemostatic factors, especially fibrinogen, found in patients with peripheral vascular disease. Smokers have lower levels of essential fatty acids and lower intakes of dietary antioxidants than non-smokers. This is due to the many reactive oxidising species in the gas and tar phases of tobacco smoke (Leng, 1995), and this may have a significant impact on smokers susceptibility to lower-limb arterial disease.

Diabetes mellitus is also believed to be a particularly important risk factor for peripheral arterial disease. Some of the increased risk may, however, be caused by the effect of diabetes on the microcirculation. Evidence relating impaired glucose tolerance (abnormal glucose tolerance test but not diabetic) in the general population to the occurrence of peripheral arterial disease is somewhat conflicting (Hughson, et al, 1978; Fowkes, 1990).

1.3 Pathology of peripheral arterial disease

In clinical practice atherosclerosis is the commonest and most important vascular disease. In common parlance, the term peripheral vascular disease is often used synonymously with peripheral atherosclerosis (Fowkes, 1990), although there are other vascular disorders that are under this heading.

1.3.1 Atherosclerosis

Atherosclerosis is a degenerative disease of large and medium-sized muscular arteries, such as the coronary, carotid, basilar and vertebral arteries, as well as the aorta and the arteries supplying the lower extremities (iliac, femoral). The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium of the artery wall. Many different cell types and a large number of growth factors, cytokines and vasoregulatory molecules are involved in atherogenesis. Atherosclerosis is the principal cause of heart attack, stroke and gangrene and loss of function in the extremities (Ross, 1993).

1.3.1.1 Lesions of atherosclerosis

The earliest lesion of atherosclerosis is the so-called 'fatty streak', an aggregation of lipid-

rich macrophages (foam cells) and T lymphocytes within the intima. Many observations have shown that fatty streaks precede the development of intermediate lesions (Masuda & Ross, 1990 [1]; [2]), which are composed of layers of macrophages and smooth muscle cells and, in turn, develop into the more advanced, complex, occlusive lesions called fibrous plaques. The fibrous plaques increase in size and, by projecting into the arterial lumen, may impede the flow of blood. They are covered by a dense cap of connective tissue matrix (including collagen, elastic fibers, and proteoglycans) with embedded smooth muscle cells that usually overlays a core of lipid and necrotic debris (Davies & Thomas, 1984; Wick et al, 1995). The fibrous plaques contain monocyte-derived macrophages, smooth muscle cells and T lymphocytes, many of which are activated, as evidenced by HLA-DR expression (Hansson, et al, 1991; Libby & Hansson, 1991).

1.3.1.2 Response to injury and atherogenesis

The exact mechanisms that lead to atherosclerosis are not completely understood. However, from knowledge accumulated over several decades by a great number of laboratories, most investigators believe that endothelial injury and the response to the injury are important factors contributing to the formation of atherosclerotic plaques (Ross & Glomset, 1973; Ross & Harker, 1976; Ross, 1993). Injury of vascular endothelium is induced in various ways, such as hypercholesterolemia, mechanical stress by high blood pressure, smoking, immune reactions, toxins, viruses and homocysteine (Dadak, et al, 1981; Wick et al, 1995). Monocytes and T lymphocytes attach to the injured sites and migrate between the endothelial cells under the influence of growth-regulatory molecules and chemoattractants released both by the altered endothelium, its adherent leukocytes and underlying smooth muscle cells (Munro & Cotran, 1988).

As the process continues, migrating cells reach further beneath the arterial surface, where the monocytes become macrophages, accumulate lipid, become foam cells and, together

with the accompanying lymphocytes, become the fatty streak (Masuda & Ross, 1990 [1]; [2]). These often form at sites of pre-existing collections of intimal smooth muscle (Thomas, et al, 1985). Thereafter, continued cell influx and proliferation lead to the more advanced lesions, distinguished by their fibrous character, and ultimately to the fibrous plaque.

Postmortem studies have shown that atherosclerosis occurs commonly in arteries serving the lower limbs and these lesions often occur close to arterial bifurcations and bends, where the mural thrombi often occur (Fowkes, 1990). It might be that haemodynamic factors, such as shear stress or disturbed flow, damage the vascular endothelium (Fowkes, 1989), causing a predisposition to platelet deposition.

1.3.1.3 Molecules and their regulation

Growth factors, cytokines and other chemicals, including lipids and small molecules such as prostacyclin and nitric oxide (NO), induce and regulate numerous critical cell functions. During the process of atherogenesis, they may act in cell recruitment and migration, cell proliferation and the control of lipid and protein synthesis (including extracellular matrix proteins). Furthermore, the same modifiers are implicated in vascular events, such as vasodilation, vasoconstriction and coagulation. Elucidation of their roles in this disease provides opportunities to develop new therapeutic agents to induce the regression of the lesions and possibly to prevent their formation.

The terms growth factor and cytokine are often used interchangeably, although the cytokines were originally considered to be the mediators implicated in immunity and inflammation and growth factors to be involved in proliferation and chemotaxis of cells in organs and tissues. With respect to atherosclerosis, these two roles are closely interrelated, with the same mediator sometimes acting as an inflammatory regulator and

sometimes acting as a growth regulator, depending on the target cell (Ross, 1993). The molecules relating to the process of atherogenesis and their biological actions are listed in Table 1-1.

Table 1-1. Molecules related the process of atherogenesis

Molecules	Source	Biological actions	References
PD-ECGF (platelet-derived endothelial cell growth factor)	Platelets	Potent endothelial mitogen	Ross, 1993
EGF / TGFα (endothelial cell growth factor / transforming growth factor α)	Platelets, macrophages	Endothelial mitogen, angiogenesis factor	Munro & Cotran, 1988; Ross, 1993
TGFβ (transforming growth factor β)	Platelets, smooth muscle cells, endothelial cells, macrophages, T lymphocytes	Inhibition of endothelial proliferation, induces gene expression of PDGF by endothelial cells and smooth muscle cells, potentially induces endothelial synthesis and secretion of connective tissue matrix, induces smooth muscle cell proliferation, endothelial and smooth muscle cell chemotaxis	RayChaudhury & D'Amore, 1991
VEGF (vascular endothelial growth factor)	Macrophages, smooth muscle cells	Endothelial mitogen	Ross, 1993

bFGF (basic fibroblast growth factor)	Macrophages, endothelial cells, smooth muscle cells	Endothelial and smooth muscle cell mitogen, induces connective tissue and smooth muscle cell proliferation, mediates angiogenic effect, chemoattractant for endothelial cells	Klagsbrun & Edelman, 1989
PDGF (platelet-derived growth factor)	Platelets, endothelial cells, macrophages, smooth muscle cells	Induces smooth muscle cell and connective tissue proliferation, chemoattractant for smooth muscle cells	Rubin, et al, 1988; Hart, et al, 1988; Ferns, et al, 1991; Ross, et al, 1990
M-CSF / GM-CSF (macrophage colony stimulating factor / granulocyte-macrophage colony stimulating factor)	Macrophages, T lymphocytes, endothelial cells, smooth muscle cells	Proliferation of T lymphocytes, endothelial cells and smooth muscle cells, maintaining cell viability and prevent apoptosis and cell death, paticipates in further macrophage activation and replication, chemoattractant for endothelial cells and monocytes, lowering of plasma cholesterol levels	Rosenfeld & Ross, 1990; Shimano, et al, 1990
HB-EGF (heparin-binding epidermal growth factor-like growth factor)	Smooth muscle cells, macrophages	Growth agonist for endothelial cells, induces smooth muscle cell proliferation	Higashiyama, et al, 1991

IGF-1 (insulin-like growth factor-1)	Platelets, endothelial cells, smooth muscle cells, macrophages	Smooth muscle cell chemotaxis, induces smooth muscle cell proliferation	Banskota, et al, 1989
IL-1 (interleukin-1)	Macrophages, endothelial cells, smooth muscle cells, T lymphocytes	Inhibiting endothelial proliferation, induces gene expression of PDGF by endothelial cells and smooth muscle cells, induces smooth muscle cell and connective tissue proliferation, activation of macrophages	Raines, et al, 1989; Wick , et al, 1995
IL-2 (interleukin-2)	T lymphocytes, macrophages	Proliferation of endothelial cells, smooth muscle cells and T lymphocytes	Ross, 1993; Wick, et al, 1995
IFN-γ (interferon-γ)	T lymphocytes,	Inhibits endothelial and smooth muscle cell proliferation	Hansson & Holm, 1991; Gray, 1992; Delomenie, et al, 1993

TNFα (tumour necrosis factor α)	Macrophages, endothelial cells, smooth muscle cells, T lymphocytes	Inhibits endothelial proliferation, induces gene expression of PDGF by endothelial cells and smooth muscle cells, induces smooth muscle cell and connective tissue proliferation, angiogenic effect mediator, activation of and chemotactic for macrophages	Old, 1985; Munro & Cotran, 1988; Jacob, 1989; Aggarwal, 1992
oxLDL (oxidised low-density lipoprotein)	Macrophages, endothelial cells, smooth muscle cells	Injury to endothelial cells, mitogenic and activating factors for macrophages underlying the endothelium, induces formation of adhesive molecule VAM-1 by endothelium, potent monocyte, T lymphocyte and endothelial cell chemotaxis	Springer, 1990; Cybulsky & Gimbrone, 1991; Leng, 1995
MCP-1 (monocyte chemotactic protein-1)	Endothelial cells, smooth muscle cells, macrophages	Potent chemoattractant for endothelial cells and monocytes	Leonard & Yoshimura, 1990

NO (nitric oxide)	Endothelial cells	Vasodilator and inhibitor of platelet aggregation, inhibits mitogenesis and proliferation of vascular smooth muscle cells	Moncada, et al, 1988; Vallance & Collier, 1994; Garg & Hassid, 1989; Luscher, 1990; Ignarro,1989[2]
PGI_2 (prostacyclin)	Endothelial cells	Vasodilator and inhibitor of platelet aggregation, cytoprotective activity.	Vane, et al, 1990; Vane & Botting, 1995;
TXA_2 (thromboxane A_2)	Platelets	Vasoconstrictor, induces platelet aggregation	Radomski, et al, 1987 (2), (3); Vane, et al, 1990
Thrombin	Plasma	Stimulates the endothelium to a procoagulant state	Ross, 1993; Lutomski, et al, 1995
Factor Xa	Plasma	Stimulates the endothelium to a procoagulant state	Ross, 1993; Lutomski, et al, 1995
Angiotensin	Plasma	Vasoconstrictor	Vane, et al, 1990

The growth-regulatory molecules can induce multiple and, in some instances, apparently divergent effects. They can stimulate cell proliferation, can in some cases inhibit proliferation, and many of the proliferative agents can act as chemoattractants (Munro & Cotran, 1988). The growth-regulatory molecules that can induce smooth muscle proliferation are generally not expressed in the normal artery, whereas they are upregulated in lesions of atherosclerosis. Chemotaxis is a critical event in the development of the lesions of atherosclerosis. It is neccesary to bring leukocytes into the artery wall and, at some sites, smooth muscle cells from the media into the intima of the artery.

Although the nature of any immune response that is induced locally during atherogenesis is not understood, it is presumed that some cytokines, such as IL-1, TNFα, IFNγ and IL-2 together with the CSFs can also play a role as immunological mediators. In the process of atherogenesis, through cell-cell interaction, the release of one molecule can lead to expression of a second molecule in a target cell that can then either stimulate its neighbours in a paracrine way, or itself in an autocrine way. IL-1, TNFα or TGFβ released by activated lesion macrophages exposed to agonists, such as oxLDL, induce PDGF-AA gene expression and secretion by smooth muscle cells (Libby, et al, 1988). Endothelial cells in culture that are exposed to the agonist IL-1 express the gene for and secrete PDGF-BB (Raines, et al, 1989).

1.3.1.4 Cellular interactions

Endothelium

Knowledge accumulated over several decades has led to the formulation of the response to injury hypothesis regarding the pathogenesis of atherosclerosis (Ross & Glomset, 1973; Ross & Harker, 1976). The different risk factors lead to endothelial dysfunction, which can elicit a series of cellular interactions that culminate in the lesions of

atherosclerosis (Wick, et al, 1995).

Research on vascular endothelium has established endothelial cells as active cells which perform a variety of critical functions (Spittell, 1992; Munro & Cotran, 1988) including: (1) provision of a nonthrombogenic surface as the endothelium linies the blood vessels in a continuous monolayer; (2) provision of a semipermeable barrier through which there is exchange and active transport of substances between blood and tissues, (3) maintenance of vascular tone by release of small molecules such as NO, which accounts for the biological activity of endothelium-derived relaxing factor (EDRF), PGI_2, and endothelin (ET) which modulate vasodilation or vasoconstriction, respectively; (4) formation and secretion of growth-regulatory molecules and cytokines; (5) ability to oxidize lipoproteins during the transgression process through endothelial cells. Changes in one or more of these properties may represent the earliest manifestations of endothelial dysfunction.

Evidence has accumulated to suggest that oxLDL is a key component in endothelial injury (Andrews, et al, 1987; Leng, 1995). Once formed by the endothelium, oxLDL may directly injure the endothelium, decreasing the production of NO and PGI_2 and thereby contributing to vasospasm and thrombosis, thus playing an initial role in the increased adherence and migration of monocytes and T lymphocytes into the subendothelial space (Wick, et al, 1995).

In atherosclerosis, the molecules mediating the attachment of platelets and circulating leucocytes to altered endothelium and the subendothelial matrix are unknown, but intensive work in the area of cell adhesion has identified several new endothelial receptors which are candidates for this adhesive anchor. These include the intracellular adhesion molecules (ICAM), which interact with specific sites on neutrophils and lymphocytes, the vascular adhesion molecules (VAM), which recognises binding sites on

lymphocytes, and the platelet-endothelial cellular adhesion molecules (PECAM), which is also expressed at high density on neutrophils and platelets. Besides facilitating leukocyte attachment, some receptors also appear to promote transendothelial migration of leukocytes. Thus, since platelets can attach to stimulated neutrophils and monocytes, platelets can be bound to the vascular wall indirectly - i.e., by adhering to leukocytes bound to the endothelium (Springer, 1990; Cybulsky & Gimbone, 1991; Ware & Heistad, 1993). The cytokines IL-1 (produced by various cells) and TNF (produced by macrophages), and oxLDL can induce the formation of these adhesion molecules on endothelium (Zimmerman, et al, 1992; Ware & Heistad, 1993; Ross, 1993).

NO is synthesised from L-arginine by NO synthase (NOS) with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor (Figure 1-1). In the endothelial cells NOS can exist in either Ca^{++} / calmodulin dependent (caNOS) or Ca^{++} independent (ciNOS) isoforms. caNOS has been suggested to be constitutive and ciNOS the inducible isoform of NOS (Moncada, et al, 1991; Li, et al, 1991). ciNOS may combine with tetrahydrobiopterin (BH_4) as a cofactor. Inducible nitric oxide synthase is expressed only after activation of cells with cytokines, such as TNFα, IFN-γ or IL-1, and lipopolysaccharide (LPS) (Moncada, et al, 1991; Li, et al, 1991; Hibbs, 1991; Zimmenman, 1992; Vallance & Collier, 1994).

The formation of NO by endothelium can prevent platelet aggregation and can cause vasodilation (Figure 1-2) (Palmer, 1987; Radomski, et al, 1987 [1] [2] [3]; Ignaaro, 1988; Luscher, 1990). NO relaxes smooth muscle and inhibits platelet adhesion and aggregation by increasing the levels of cyclic guanosine monophosphate (cGMP). Vasomotor tone of the artery appears to be controlled by the constant action of NO (Ress, et al, 1989 [1] [2]; Vallance, et al, 1989; Panza, et al, 1990; Tolins, et al, 1990; Tolins & Shultz, 1994). Inhibition of formation of NO or eicosanoids, such as PGI_2, permit the opposing forces of vasodilation, resulting from vasoconstrictors such as ET,

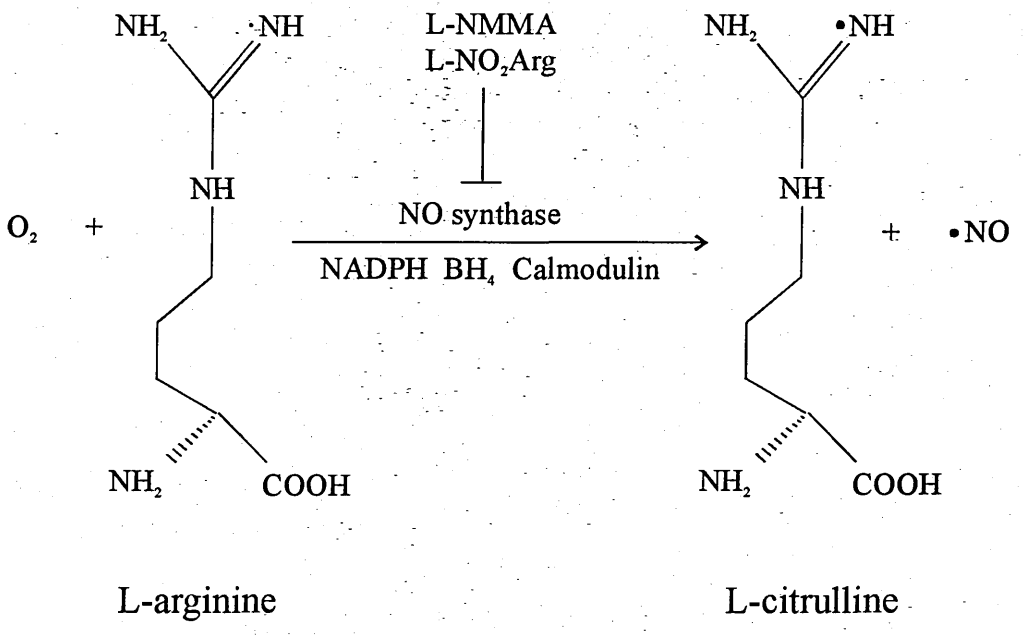


Figure 1-1. The conversion of L-arginine to reactive nitrogen intermediates.

NO synthesis is selectively catalysed by NO synthase and is competitively inhibited by L-arginine antagonists, L-NMMA (N^G-monomethyl-L-arginine) or L-NO_2Arg (N^W-nitro-L-arginine).

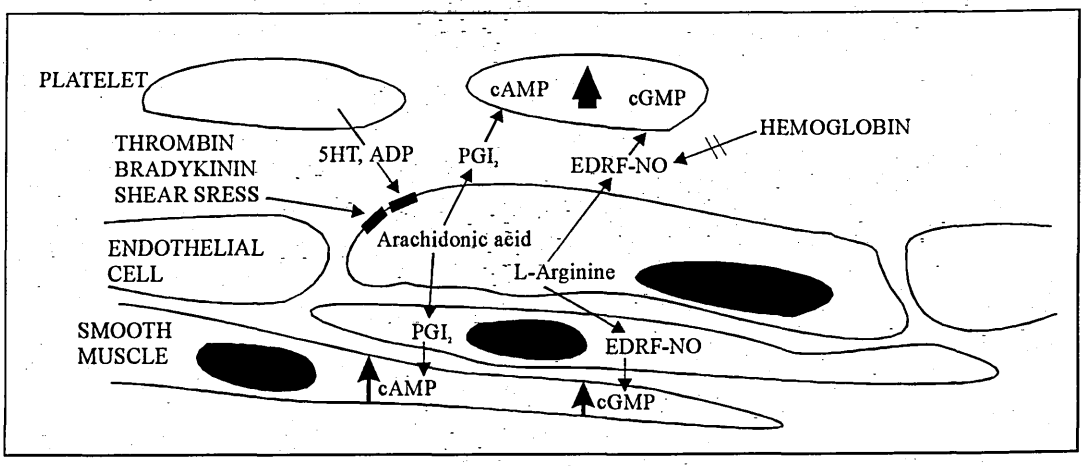


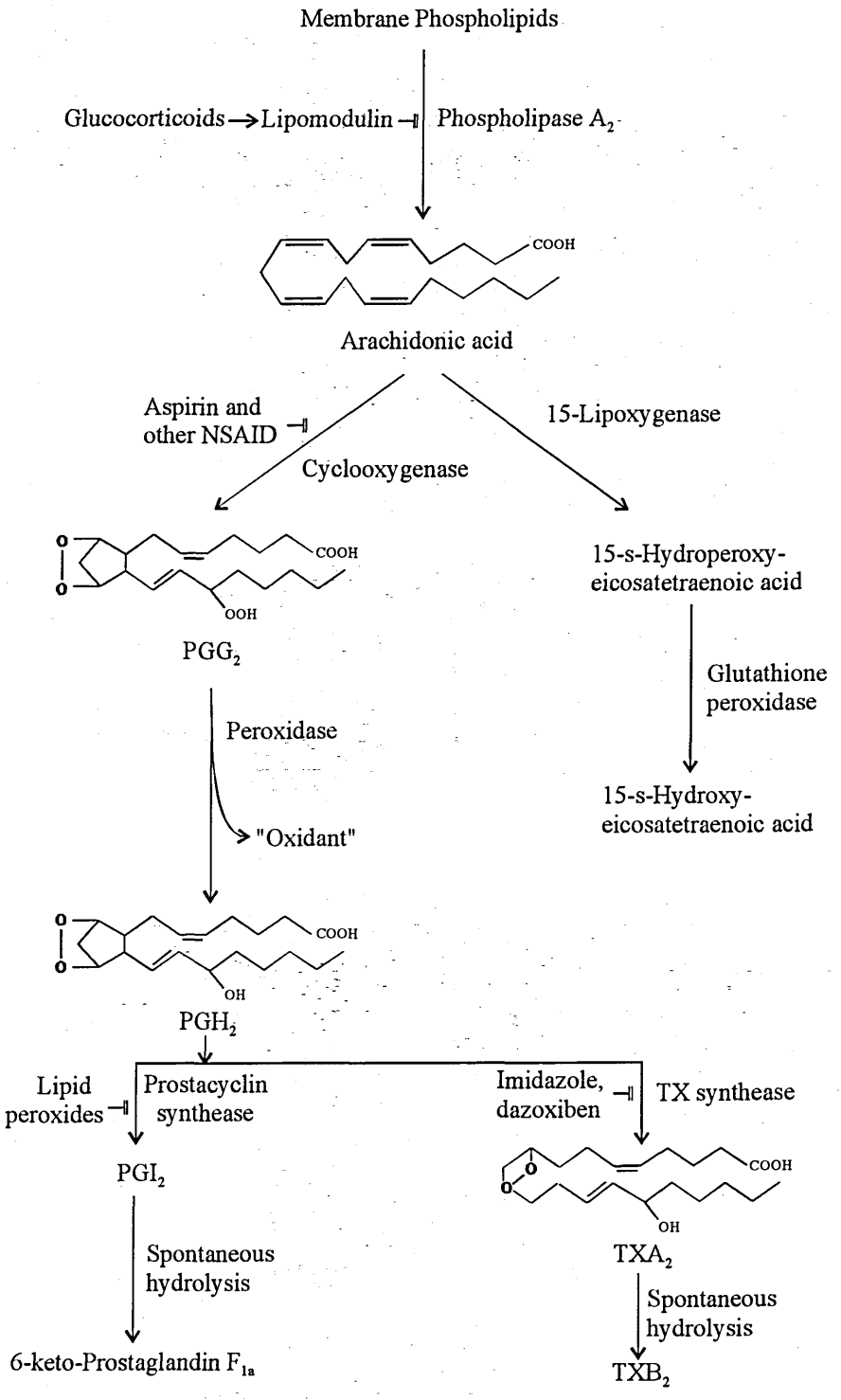
Figure 1-2. Generation of prostacyclin (PGI_2), EDRF-nitric oxide in normal endothelial cells (Vane et al, 1990).

Stimulation of receptors on the cells by serotonin (5HT) or adenosine diphosphate (ADP) released from platelets or by thrombin, bradykinin, or shear stress leads to the release of vasoactive mediators. Prostacyclin formed from arachidonic acid relaxes smooth muscle and inhibits aggregation of platelets by increasing levels of cyclic adenosine monophosphate (cAMP). EDRF-nitric oxide (NO) formed from L-arginine also relaxes smooth muscle and inhibits platlet aggregation and adhesion, by increasing levels of cyclic guanosine monophosphate (cGMP). By increasing cAMP and cGMP simultaneously, prostacyclin and EDRF-nitric oxide synergistically inhibit platelet aggregation. Haemoglobin inactivates EDRF-nitric oxide.

angiotensin II (A-II), or thromboxane A_2 (TXA_2) (Vane, et al, 1990).

The endothelium plays a critical role in maintaining the fluidity of blood as well as the patency of the blood vessels. Heparan sulphate, PGI_2 and NO produced by endothelial cells are all antithrombotic (Vane, et al, 1990; Ware & Heistad, 1993) and endothelium can specifically bind factors that prevent coagulation (Rau, 1991; Spittell, 1992). In blood platelets, arachidonic acid is metabolised through a complex pathway to TXA_2, which exerts an entirely opposing physiological effect on the cardiovascular system to PGI_2. It has been suggested that the balance between these substances may have an important controling influence in thrombosis (Radomski, et al, 1987 [1]; [2]; [3]). Careful histological studies of athromatous lesions in both human and animals have indicated that fibrin and platelets are important constituents of early lesions (Ross & Glomset, 1973). Any factor that predisposes towards platelet aggregation would have an important influence on the development of early atheromatous lesions.

PGI_2 is generated mainly by the endothelium (Figure 1-3) and its synthesis can be stimulated by endothelial contact with activated leukocytes (Vane, et al, 1990). *In vitro*, human umbilical vein endothelial cells have been used to study the effects of the interaction between human monocytes and endothelial cells on endothelial PGI_2 production. Hakkert and his colleagues (1992) have reported that direct cell-cell contact between purified monocytes and endothelial cells strongly enhanced PGI_2 synthesis. Secretory products, such as IL-1, from monocytes and neutrophils and TNFα, from activated monocytes/macrophages, also enhanced endothelial PGI_2 synthesis (Vane & Botting, 1995), although to a lesser extent than with monocytes that were in direct contact with endothelial cells. The explanation may be that monocytes also produce and secrete arachidonic acid metabolites by the cyclooxygenase pathway. Thromboxanes are the major products synthesized by monocytes via this route, whereas PGI_2 is produced only in small amounts. It is possible that monocytes in close contact with endothelial



PG, prostaglandin; TX, thromboxane; NSAID, nonsteroidal anti-imflammatory drug; ⊣, inhibits.

Figur 1-3. Metabolic pathway showing formation of PGI_2 and TXA_2 from arachidonic acid in endothelial cells

cells enhance the endothelial PGI_2 production by providing the endothelial cells with exogenous eicosanoids (Hakkert, et al, 1992).

Nitric oxide production by the endothelium may also be stimulated by peripheral blood cells. *In vitro* studies in the rat have shown that endothelial cells co-cultured with activated lymphocytes showed an increase in production of nitric oxide, this effect being dependant on cell-cell contact (Schuler, et al, 1995). As nitric oxide is known to inhibit leukocyte: endothelial cell interaction (Kuber, 1993), these may be local feedback control which regulates the adhesion of leukocytes to endothelial cells.

Platelets

The earliest and most consistent consequence of denuding endothelial injury is the adhesion of platelets to the subendothelial connective tissue followed by the platelet release reaction (Longenecker, 1985). The biologically important molecules released from platelets include β-thromboglobulin, vasoconstrictive amines, arachidonic acid metabolites, and, of most relevance to atherogenesis, mitogenic factors PDGF, TGFα and TGFβ (Table 1-1). PDGF induces both migration and proliferation of smooth muscle cells.

Platelets are excellent candidates as the initiators of intimal plaque formation after denuding injury. In the human, it is highly likely that platelets are involved in early lesion formation in catheter-induced injury, in peri-anastomotic lesions of vascular grafts, and in other traumatic vascular lesions (Ferrell, 1992).

Monocytes / macrophages. Macrophages are present in all stages of atherosclerosis. The roles of the macrophages include: (1) action as an antigen-presenting cell to T lymphocytes, (2) action as a scavenger cell to remove antigenic materials, (3) production of growth-regulatory molecules and cytokines. The macrophage is thus the principal

inflammatory mediator of cells in the atheromatous plaque microenvironment.

LDL is a possible candidate antigen and may be taken up by the macrophages that transform into foam cells. Macrophages can oxidize LDL into oxLDL, which could set up a vicious cycle that could further exacerbate the atherogenic process (Leng, 1995). Macrophages also play a role in the fibroproliferative process by their capacity to form numerous growth factors, in particular PDGF (Ross, et al, 1990) as well as IL-1 and TNFα (Table 1-1). PDGF exerts a mitogenic effect on mesenchymal cells and stimulates leukocyte migration, thus playing a role in sustaining an inflammatory / immune response (Wich, et al, 1995).

Smooth muscle. These cells respond to agents that induce either vasoconstriction, such as ET, A-II or vasodilation, such as PGE, PGI_2 and NO when cells are in a contractile phenotype. They are capable of expressing genes for a number of growth factors and cytokines, can respond to growth factors by expressing appropriate receptors, and can synthesize extracellular matrix. At sites of atherosclerosis, damaged smooth muscle cells may release FGF and in so doing could also stimulate neighbouring smooth muscle, the overlying endothelium, or vascular channels within the lesions. Their synthetic activity will determine the matrix content of the lesion, which in turn could interact at their surface and modify their capacity to respond to various agonists. Thus the smooth muscle cell plays the critical role in the fibroproliferative component of this disease process.

1.3.1.5 Immune responses

The involvement of immune reactions, at all stages of atherosclerotic development (Libby & Hasson, 1991), has been neglected for many years in classical atherosclerosis research. It is now evident that T cells are among the earliest cells infiltrating the arterial

intima in the initial stages of atherosclerosis. In the T-cell population, 70% were found to be CD_4+, the remainder being CD_8+, and the majority expressed major histocompatibility complex (MHC) class II HLA-DR and IL-2 receptor (IL-2R), thus indicating an activated stage (Wick, et al, 1995).

The presence of T lymphocytes together with numerous activated macrophages suggests that not only is there an inflammatory reaction during the process of atherogenesis, but also that there may be an immunological reaction and that this may be related to a specific antigen (Ross, 1986; Munro & Cotran, 1988). Although it has not been possible thus far to identify such an antigen, autoantibodies have been found to oxLDL. Furthermore, clonal expansion of lymphocytes does not appear to take place (Libby & Hasson, 1991). Whatever antigen the T cells that infiltrate the arterial intima are recognizing, it seems improbable that endothelial cells aberrantly expressing MHC class II antigens act as primary T-cell sensitization sites: HLA-DR, -DP and -DQ expression by endothelial cells was never observed without concomitant subendothelial accumulation of mononuclear cells capable of producing IFN-γ, pointing to MHC class II expression as a secondary phenomenon (van der Wal, et al, 1989). Thus, T-cell sensitization must occur at another site such as the regional lymph nodes. This suggestion is also supported by the demonstration of increased serum levels of neopterin, reflecting systemic activation of macrophages by IFN-γ (Tatzber, et al, 1991). In the studies on the immune system in the process of atherogenesis numerous questions remain to be answered.

1.3.2 Hypertensive vascular disease

Hypertension accelerates atherosclerosis (Chobanian et al, 1989) and the lesions have the same histological appearances and distribution as normotensive subjects with atherosclerosis (Haudenschill et al, 1979; Limas et al, 1982; Huttner & Gabbiani, 1983;

Chobanian et al, 1986). However, hypertension also causes thickening of the media of the muscular arteries (Spittell, 1992). This is the result of hyperplasia of smooth muscle cells and collagen deposition close to the internal elastic laminae. In contrast to artherosclerosis, which affects larger arteries, it is the smaller arteries and arterioles (vessels with an outside diameter of less than 100 μm) which are especially affected in hypertension.

1.3.3 Diabetic vascular disease

Diabetic vascular disease is associated with damage involving both large and small vessels throughout the body. Patients with diabetes, particular juvenile - onset insulin-dependent diabetes, may develop three forms of vascular disease:

- Atherosclerosis.
- Hypertensive vascular disease.
- Capillary micro-angiopathy. This is the most important and characteristic change in diabetes. The alterations are found throughout the systemic circulation and can be viewed directly in the retina. Small arterioles and capillaries are affected and there are both basement membrane thickening and intimal fibrosis.

1.4 Current pharmacological approaches to the treatment of peripheral arterial disease

Many elderly people with intermittent claudication may well not seek medical care, but for those who do, clinical medicine has little to offer, especially if symptoms are mild to moderate.

Recently clinical pharmacological approaches in the treatment of PVD mainly involves the management of thrombosis and administration of vasodilators.

1.4.1 Anticoagulants

Oral anticoagulants e. g. warfarine and dicoumarol, are used to prevent thrombus formation or the extension of an already existing thrombus in the slow-flow, low pressure venous side of the circulation, where the thrombus consists of a fibrin web enmeshed with platelets and red cells. Oral anticoagulants are therefore widely used in the prevention and treatment of deep-vein thrombosis in the legs. However, anticoagulants are of less use in preventing thrombus formation in arteries, for in the high-pressure, high-velocity arterial system thrombi are composed mainly of platelets with little fibrin (the 'white' thrombus) and a coagulopathy due to deficiency of natural occuring anticoagulants (antithrombin III, protein C and protein S) producing arterial thrombosis seems very unlikely (Cooke, 1995). The serious adverse effect of anticoagulants is haemorrhage.

1.4.2 Thrombolytic agents

Thrombolytic agents, such as streptokinase, act by activating plasminogen to the proteolytic enzyme plasmin, which degrades fibrin and other procoagulant proteins including factors V and VIII and so digests thrombi. Streptokinase has only a limited success rate, with serious complications such as bleeding, hypotension and stroke (Lutomski, et al, 1995) and is not recommended (Housley, 1988).

1.4.3 Antiplatelet agents

By decreasing platelet aggregation, antiplatelet agents may inhibit thrombus formation on the arterial side of the circulation, where thrombi are formed by platelet aggregation and anticoagulants have little effect. Antiplatelet drugs have little effect in venous

thromboembolism.

Aspirin irreversibly inhibits prostaglandin G / H synthase (cyclooxygenase) by acetylation of the hydroxyl group of a single serine at its active site. Endothelial cells are nucleated and produce cyclooxygenase, regaining their ability to regenerate prostacyclin production in a few hours. However, non-nucleated platelets cannot produce cyclooxygenase, and thromboxane synthesis recovers only as new platelets enter the circulation. The life of a platelet is about 8 to 11 days. Thus, treatment with low dose aspirin leads to a cumulative inhibition of thromboxaneA_2 formation and allows endothelial cells to produce prostacyclin (Figure 1-3) by newly generated synthesised enzyme. Several administration methods and dosage forms of aspirin have been suggested so that the effects of aspirin on platelet cyclooxygenase will be optimised and those on endothelial cell (vasculature) cyclooxygenase will be minimised (Lutomski, et al, 1995).

An overview of trials of antiplatelet treatment in patients with transient ischaemic attacks or minor ischaemic strokes (as in patients with unstable angina or myocardial infarction) showed that 15% fewer treated patients died and 30% fewer had non-fatal strokes or myocardial infarctions and that there is no evidence to suggest benefit in PVD (Lowe,1990). Because atherosclerosis is a generalised disease and patients with PVD have an increased risk of myocardial infarction and transient ischaemic attacks, physicians may consider using aspirin (Cooke. 1995).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed, but have the potential for serious side effects, particularly the risk of gastric irritation and slight but persistent blood loss, especially in the elderly. The sudden onset of exertional dyspnoea may be the first indication of side effects (British National Formulary, 1994).

1.4.4 Vasodilator drugs

Prostacyclin (PGI_2, epoprostenol) and its analogue iloprost are potent vasodilators and inhibit platelet aggregation by increasing cAMP in platelets and vascular smooth muscle cells. Intravenous prostacyclin and iloprost are already of proven value in salvaging ischaemic limbs when surgery other than amputation may not be possible. These two drugs must be given by continuous intravenous (iv) infusion (Grant & Goa, 1992), which must be given continuously for at least 3 days or longer in the treatment of vascular diseases, including thromboangitis obliterans and Raynaud's phenomenon.and primary pulmonary hypertension (Belch et al, 1981, 1983; Szczeklik et al, 1979; Jones et al, 1987; Vane & Botting, 1995). Side-effects are dose dependent and include flushing, headache, gastrointestinal distress and hypotension, with bradycardia, pallor and sweating at higher doses.

Nitrate formulations have the active moiety of nitric oxide (Feelisch, 1991). Nitric oxide induces relaxation of vascular smooth muscle cells and inhibition of platelet aggregation by increasing intracellular cGMP. Nitrate formulations have an important role both for prophylactic use (taken before exertion) and for angina occurring during exercise or at rest. Nitrovasodilators are known to act in heart failure (Duprez & Clement, 1992) either by arteriolar dilatation which reduces both peripheral vascular resistance and left ventricular pressure at systole resulting in improved cardiac output, or by venous dilatation reducing cardiac pre-load. Nitrate formulations have no place in treating peripheral ischaemia from atherosclerosis, they have no effect on symptoms and may in theory be harmful because flow may be diverted to dilated unaffected vesscls from the affected vessels ("steal" blood phenomenon). Adverse effects include flushing, headache and postural hypotension.

Slow channel calcium blockers, such as nifedipine reduce the frequency and severity of

attacks, even in organic change such as Raynaud's phenomenon associated with systemic sclerosis and vasospasm. Side-effects include headache, flushing, dizziness, lethargy; also gravitational oedema, rash, nausea, increased frequency of micturition, eye pain, gum hyperplasia; depression and telangiectasia (British National Formulary, 1994).

Alpha adrenoceptor blockers such as prazosin and doxazosin may produce unacceptable hypotension. Beta adrenoceptor blockers, such as atenolol, which are usually used in treating hypertension, should be avoided because of their vasoconstrictive action on peripheral vessels due to unrestricted release of the peripheral sympathetic system (Cooke, 1995).

Giving up smoking may be of benefit in ameliorating progression of disease and is essential in conservative management. This may be due to beneficial changes in haemostatic and rheological factors rather than regression of atherosclerosis (Fowkes, 1990). Stopping smoking in conjuction with exercise, appropriate weight reduction and lowering of blood cholesterol (by diet or lipid lowering drugs, such as gemfibrozil, in those with levels of 8 mmol cholesterol / L or over) may stablise the disease, enhance general health and may so improve claudication distance that the patient may become asymptomatic. The main effect of exercise may be to dilate collateral vessels and increase blood flow by reducing viscosity (Housley, 1988).

1.5 Alternative approaches to drug therapy

For decades many drugs have been used for the treatment of vascular disease, but drug therapy has not successfully decreased the mortality and morbidity of peripheral vascular disease.

Autohemotherapy, treatment of blood *ex vivo* followed by its re-injection, with blood

treated with medical ozone alone or in combination with heat and ultraviolet light has been practised in parts of Western Europe for many years (Rilling & Viebahn, 1987). By 1986, no less than 340,000 treatments had been performed without any untoward effects (Bocci, 1994). A number of claims have been made for such treatments, particularly an improvement in peripheral vascular disorders (Riva, et al, 1990). Immunological effects have been suggested by claims of benefit in viral diseases (Bassi, et al, 1982) and in some malignant tumours (Hernuss, 1975).

Regretfully, this form of autohaemotherapy has been carried out in an empirical fashion without appropriate controls, particularly in the treatment of viral diseases. The underlying mechanisms resulting in the clinical improvement in patients are not understood. However, there appears to be sufficient subjective anecdotal evidence to warrant scientific investigation, which is the purpose of this research.

As an introduction to the application of ozone in medicine, the immune system effects of ozone will be reviewed. Because ozone may be used in combination with heat and ultraviolet irradiation, the effects of these two stimuli on the human immune system will also be discussed.

1.5.1 The use of ozone in medicine

During the First World War, the healing of wounds and fistulae with ozone and oxygen gas mixtures was reported sporadically, and a medical use was suggested.

Ozone can be formed by the action of UV light on oxygen. Conversely, the ozone molecule is capable of absorbing UV to produce diatomic oxygen once more. Ozone is the triatomic allotrope of oxygen and one of several forms of the oxygen atom which exist in nature. It is an extremely potent oxidant, unstable in an aqueous environment.

Research on the effects of inhaled ozone has been intense. Ozone is highly toxic to pulmonary tissue and causes irreversible morphological damage which leads to interstitial fibrotic changes with associated decrements in pulmonary function when inhaled as an environmental pollutant (Hatch, et al, 1990). It has a broad spectrum of antimicrobial activity, killing bacteria, fungi, and viruses (Rilling & Viebahn, 1987; Wells, et al, 1991; Carpendale & Freeberg, 1991).

The powerful disinfectant property of ozone gas has been used in medicine, the gas being administered at the surface using a pneumatic cuff or similar device. Medical ozone, a mixture of ozone and oxygen, has been used in the treatment of various diseases, including the disinfection of open wounds and ulcers. Relief of pain has been noted in addition to its fungicidal, virucidal and bactericidal action. The resulting acceleration of healing has been attributed to an improvment of the oxygen supply to hypoxemic tissue areas (Dolphin & Walker, 1979).

For systemic diseases, such as circulatory disorders and viral infections, some form of invasive route of administration is required. To minimise the potential harmful side effects of injecting ozone gas directly, treatment of blood *ex vivo* followed by its re-injection (autohaemotherapy) has been a route widely adopted in ozone therapy. Minor autohaemotherapy in which 5-10 ml of blood is treated with about 100 μg of ozone and major autohaemotherapy in which 50-100 ml of blood is treated with about 2000 μg of ozone are approaches that have been used (Rilling & Viebahn, 1987; Garber, et al, 1991; Romero, et al, 1993 [1] [2]).

There are a number of publications, claiming benefit for autohaemotherapy with ozonized blood .

In 1993, Romero and his colleagues observed 72 non-diabetic patients with

atherosclerosis obliterans, Fontaine stage II (intermittent claudication), treated by intravenous (major autohemotherapy) and intramuscular (minor autohemotherapy) injections. They claimed that there was an improvement in the ozone treated group in comparison with a control group treated by conventional means, the claudication distance in the treadmill test increased to 2.5 Km / hour (Romero, et al, 1993 [1]). Turczynski, et al (1991[1] [2]) reported that patients with diabetes mellitus and coexisting symptoms of obliterative atherosclerosis of the lower limbs treated with ozonized blood administrated by major autohemotherapy had a similar improvement. In another small group of 15 patients with atherosclerosis obliterans of the lower limbs treated with ozonized blood autohemotherapy, Romero and his colleagues stated that there was a statistically significant decrease in amputation ratio (26.7 %) and surgery for patients with rest pain (13.3 %) in comparison with the control group (46.7 and 26.7 % respectively) (Romero, et al, 1993 [2]). Sroczynski et. al., observed 50 patients with atherosclerotic ischemia of the lower extremities and 49 diabetic patients treated with ozonized blood using major (intravenous) autohemotherapy. The treatment showed a significant improvement in both groups manifested by an increase in ankle-brachial index, and a doubling of the intermittent claudication walking distance (Sroczynski et al, 1992). Kawalski, et. al. (1992) reported that 25 patients were given major autohemotherapy with ozonized blood pre- and post-rhinoplasty, resulting in a significant reduction in post-operative complications as compared to the control group.

Gierek et al (1992) reported on 206 patients with ophthalmologic disorders, such as inflammatory corneal ulcerations, optic neuritis and retinal pigment dystrophy that were treated by major autohemotherapy with ozonized blood. These patients had an improvement of visual acuity and in visual fields. Riva and his colleagues (1990) observed 20 patients affected by age-related degenerative maculopathy. The results have indicated that the majority of patients showed an improvement of their ocular condition confirmed by visual acuity and eye fluoroangiography after major autohemotherapy with

ozonized blood.

Autohemotherapy with ozonized blood also has been successful used in treatment of acute and chronic viral diseases, such as herpes (healing of skin lesions and keratitis) and hepatitis and neoplasms (Rilling & Viebahn, 1987; Bassi et al, 1982).

1.5.2 The effects of ozone on the immune system

The potential effects of treating whole blood with ozone include the production of a number of powerful reactive oxygen species, the peroxidation of phospholipids with the consequent disruption of cell membranes and modification of specific receptors on cell membranes, leading finally to lymphocyte mitogenesis with possible induction and release of lymphokines (Bocci & Paulesu, 1990). It is known that several types of oxidants, such as periodate, hydrogen peroxide and galactose oxidase, could also trigger the synthesis and release of interferon and other cytokines from monocytes and lymphocytes.

Hydrogen peroxide and its biosynthetic precursor superoxide anion (O^{2-}) stimulate a strong increase in expression and synthesis of interleukin-2 by accessory cell-depleted T-cell populations (Roth & Droge, 1989). Munakata and his colleagues have demonstrated that both exogenous and endogenous hydrogen peroxide released from activated macrophages induce the production of interferon and enhance natural killer (NK) cells activity. Both exogenous and endogenous hydrogen peroxide may be regarded as inducing IFNγ (Munakata et al, 1985).

Dianzani et al have shown that human lymphocyte cultures produce large amounts of interferon after treatment with the enzyme galactose oxidase (Dianzani et al, 1979). A novel monokine MBF (macrophage-derived blastogenic factor) is secreted from human macrophages stimulated with galactose oxidase. This monokine stimulates resting T

lymphocytes to produce IFNγ and to proliferate (Antonelli et al, 1988).

The oxidation of cell surface structures by mild oxidation with sodium periodate also induces marked polyclonal proliferation of peripheral blood mononuclear cells (PBMC) and causes the production of IFNγ (Antonelli et al, 1988).

The specific effects of treating blood *in vitro* with oxygen containing small concentrations of ozone include the stimulation of production of IFNγ (Bocci & Paulesu, 1990) and tumour necrosis factor-alpha (TNF-α) from human leukocytes (Paulesu et al, 1991). Recently it has been reported that oxygen / ozone induces the production of other cytokines, such as IL-1β, IL-2, IL-6, IFNβ and GM-CSF, by PBMCs (Bocci, et al, 1994).

The oxidation of galactose residues on cell surface structures is considered a general feature of lymphocyte activation whatever the inducer (Antonelli et al, 1988; Dianzani et al, 1979). Cells may respond to extracellular stimuli by the de novo synthesis of proteins. Experimental evidence suggests that NF (nuclear factor)-kB is an oxidative stress-responsive transcription factor of higher eukaryotic cells (Schreck et al, 1992). NF-kB can activate a great variety of genes involved in early defence reactions of higher organisms. The agents which can target NF-kB activation include the cytokines interleukin-1 and tumor necrosis factor, viruses, double-stranded RNA, endotoxins, phorbol esters, ultraviolet and ionizing irradiation and reactive oxygen intermediates (ROI), such as low concentrations of H_2O_2 (Schreck et al, 1992).

NF-kB is a member of a novel family of transcription factors and is a multiprotein complex with 50kD (p50) and 65kD (p65) components. In nonstimulated cells, NF-kB resides in the cytoplasm in an inactive complex with the inhibitor IkB. Stimulation by reactive oxygen intermediates cause release of IkB and allows NF-kB to enter the

nucleus and bind to DNA control elements thereby inducing the synthesis of mRNA (Figure 1-4). NF-kB may provide an important model system to explore a potential role of oxidative stress in intracellular signalling processes.

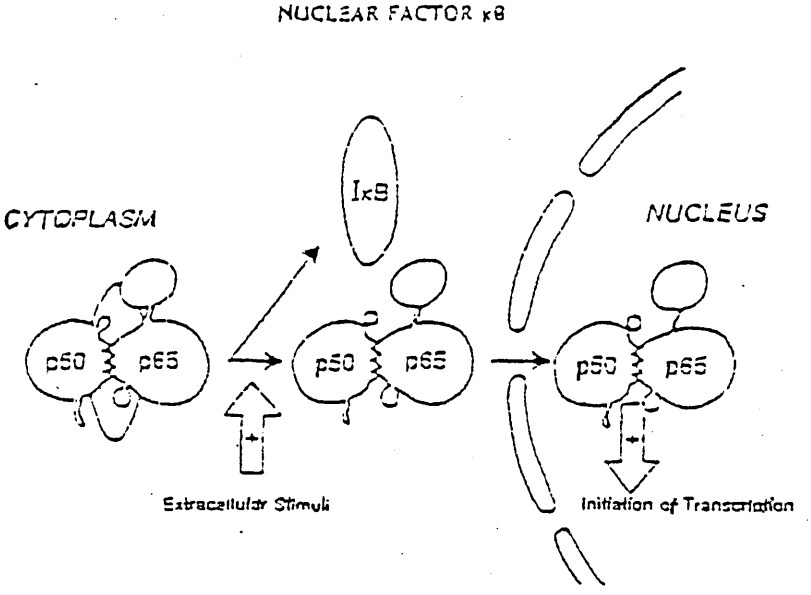


Figure 1-4. The activation of NF-kB.

1.5.3 The effects of ultraviolet radiation on the immune system

The UV region of the electromagnetic spectrum is divided into three classes: UVA (320 - 400 nm), UVB (290 - 320 nm) and UVC (200 - 290 nm). The shortest wavelengths have the highest energy. UVC has been termed germicidal radiation, since it is effective in killing single-celled organisms.

UV irradiation has profound immunomodulatory effects. It has been reported that UV irradiation has marked effects on the production of cytokines from mouse lymphocytes: the secretion of IL-3, IL-6, tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) is induced by UV irradiation (Pamphilon, et al, 1991).

Mirando and colleagues have demonstrated that monocytes irradiated *in vitro* with ultraviolet light efficiently inhibited the intracellular replication of mycobacterium avium intracellulare (MAI). Ultraviolet radiation augmented monocyte antimicrobial activity for MAI in a dose-dependent fashion (Mirando, et al, 1992). The inhibition of MAI growth was thought to be mediated via a modulation of cell-mediated immunological function by UV exposure. UV radiation triggers the synthesis of TNF-alpha from monocytes.

However, comparing the absorption spectra of biological molecules with the action spectra of ultraviolet radiation-induced events suggests that deoxyribonucleic acid (DNA) may be the target for UV light (McKenzie & Sauder, 1994). Suzuki and Watanabe (1992) have reported that there is an augmented gene expression of HSP (heat shock protein) 72 in normal human cells after irradiation by ultraviolet light.

1.5.4 The effects of heat on the immune system

The heat shock response is one of the most universal reactions known and heat shock proteins (HSPs) are among the most conserved molecules in phylogeny. HSPs are produced when a prokaryotic or eukaryotic cell is confronted with a sudden increase in temperature (Kaufmann, 1990). When it was found that many insults other than heat can also induce HSP synthesis the term 'stress proteins' was introduced. *In vitro* after a short exposure (usually 20 minutes) to 41°C - 43°C, human alveolar macrophages are induced to synthesize 70 and 83 kDa HSPs, but inhibition of the synthesis is apparent after exposure to 37°C or 45°C (Polla, 1988).

It has become increasingly clear that HSPs can modify the function and destiny of other proteins and thus play an important role in numerous physiological processes.

HSPs are grouped into families of different molecular sizes. Members of a given family share several features in addition to size. In mammalian cells, HSP60 catalyses the folding of mitochondrial proteins. HSPs may accelerate the removal of denatured proteins inside a cell. These features have sometimes been called 'house-keeping functions' of HSPs. One of the best-studied examples of this is found in immunology: the heavy chain binding protein, Bip, which participates in the assembly of immunoglobulins belongs to the HSP70 family. Bip binds to the immunoglobulin heavy chain within the endoplasmic reticulum until association with the immunoglobulin light chain occurs, and thus prevents premature self assembly of heavy chains. In the absence of light chains, Bip remains permanently bound to the heavy chain, retaining it within the lumen of the endoplasmic reticulum. HSPs are involved in immunity and the immune system has adopted certain HSPs for its own particular purposes. Some HSP genes are localised within the major histocompatibility complex (MHC). Genes encoding HSP70 have been mapped in the human MHC between the loci for complement components and

tumour necrosis factor.

Stress can be induced by different stimuli including heat, ultraviolet irradiation, viral infection and oxygen metabolites, such as H_2O_2 (Polla, 1988; Kaufmann, 1990; Suznki & Watanabe, 1992).

1.6 Conclusion

Peripheral vascular disease is responsible for considerable mortality and morbidity in heart attack and stroke, and is the principal cause of gangrene and loss of function of the extremities. Currently no successful treatment has been achieved for peripheral vascular diseases. The atherosclerosis process is the principal contributor to the pathogenesis of peripheral vascular disease. The process, in normal circumstances a protective response to insults to the endothelium and smooth muscle cells of the wall of the artery, consists of the formation of fibrofatty and fibrous lesions, preceded and accompanied by inflammation. With the progression of the process the artery concerned becomes narrow or occluded and this results in impairment of the blood supply. Many different cell types and a large number of growth factors, cytokines, regulators of platelet aggregation and vasoregulatory molecules participate in this process. Adjusting the expression of genes encoding these molecules and the targeting of specific cell types provides opportunities to develop new therapeutic agents to induce the regression of the lesions and, possibly, to prevent their formation. The elucidation of the roles of different cell types and the various molecules that are involved in atherogenesis provide an opportunity to investigate the mechanisms of HOU-autohaemotherapy.

1.7 Aims of study

To determine the *in vitro* and *in vivo* biological changes occurring as a result of the treatment of blood with heat, ozone / oxygen and ultraviolet light (HOU), and to provide a scientific explanation for the many claims made for autohaemotherapy in a number of human disorders and pathologies.

(1) To investigate the effect of treatment of blood with HOU on blood platelet activity *in vitro* in order to gain an understanding of how such autohaemotherapy might resolve peripheral vascular disease.

(2) To investigate the effect of treatment of blood with HOU on immune cells in peripheral blood *in vivo* in order to gain an understanding of how such autohaemotherapy might be beneficial in the treatment of viral diseases, immunodeficiencies and tumours.

(3) To investigate changes in levels of immune and haematological mediators in blood following treatment, in order to gain an understanding of how autohaemotherapy might result in effects manifested throughout the body.

CHAPTER 2

The Effects of *in vitro* Treatment of Blood with Heat, Ozone/Oxygen and UV Irradiation on Blood Platelets

2.1 Introduction

In spite of the fact that autohaemotherapy with ozone- or HOU-treated blood has already been carried out with beneficial results in peripheral vascular diseases, the mechanism of action remains speculative and uncertain. There is increasing evidence that thrombosis is important in the development and progression of peripheral vascular disease (PVD) (Ross, 1993; Lowe, et al, 1994) and that platelets are one of the important constituents of thrombosis. The effect of treatment with HOU of blood from healthy volunteers and patients with PVD and diabetes *in vitro* on platelets and the mechanisms of the action were investigated in order to gain an understanding of how such autohaemotherapy might resolve peripheral vascular disease. *In vitro* data on the effect of this treatment on platelet function are currently lacking.

2.2 Materials and methods

The reagents and equipment used are listed in Appendix 4-3.

I *In vitro* study in healthy volunteers

2.2.1 Blood sample collection

Venous blood (20 ml), taken from the healthy non-smoking volunteers, aged between 20-50 years, was collected into tubes containing 3.8 % sodium citrate anticoagulant in a ratio of blood to anticoagulant of 9:1. None of the volunteers had taken any medication

for at least 1 week prior to the investigation. The blood was divided into two 10 ml aliquots. One aliquot was treated with heat, ozone / oxygen and ultraviolet (HOU) (see below), the other was used as an untreated control sample.

2.2.2 Treatment of blood samples

Blood was treated with different concentrations of ozone using the Ozon-O-Med equipment (Appendix 2-1). In this system, blood is raised to and maintained at a temperature of 42.5°C and is exposed to ultraviolet light at a wavelength of 253.7 nm (Muller & Jentiens, 1990). Ozone in medical oxygen is bubbled through the blood at a oxygen flow rate of about 0.12 L / min for 3 mins. The concentration of ozone is variable between 5 and 50 μg / ml and is measured using an ozone monitor.

2.2.3 Reagents for the study of platelet aggregation

The reagents for the study of platelet aggregation included ADP, collagen, thrombin, calcium ionophone A23187 and adrenaline. For the preparation of these reagents see Appendix 4-3-3.

2.2.4 Platelet aggregation in HOU-treated whole blood

In order to investigate whether the HOU-treatment whole blood had an effect on platelet aggregation, platelet aggregation in HOU-treated whole blood was studied.

Platelet aggregation was measured by an end point turbidometric method (Born, 1962).

To prepare platelet-rich-plasma (PRP), 10 ml aliquots of blood sample (either HOU-treated or untreated control blood) was centrifuged at 200 xg for 15 mins at room

temperature (18-25°C). The PRP was carefully removed using a plastic transfer pipette.

PRP (0.225 ml) was placed in an aggregometer cuvette and equilibrated to 37°C in the heating block of the instrument. The speed of the stirrer bar was 1,100 rpm. After stirring was commenced, 0.025 ml of platelet aggregation agent was added at the final concentrations indicated in the results section. Light transmission through the plasma was monitored continuously on a chart recorder. The addition of an aggregating agent results in the formation of increasingly larger platelet aggregates with a corresponding decrease in optical density. The change in optical density was recorded on the chart recorder. Each aggregation curve was observed for up to 3 minutes. Platelet aggregation in the HOU-treated samples was expressed as the percentage of the aggregation in an untreated control samples for each individual experimental condition.

2.2.5 Washed platelet aggregation studies

In order to investigate whether HOU-treatment of whole blood has a direct or an indirect effect on platelets via production of the factors affecting platelet aggregation in whole blood, aggregation of washed platelets was studied.

Blood (20 ml) was collected from each of 5 normal healthy individuals. One 10 ml aliquot was treated with heat, ozone/oxygen and UV as described above, the other 10 ml aliquot was used as an untreated control. PRP was prepared from both these samples. Platelets and PPP were prepared from the PRP by centrifugation at room temperature for 10 minutes at 1500 xg. The platelet poor plasma (PPP) was removed from the sedimented platelets using a plastic transfer pipette. The platelets were washed 4 times in PBS by centrifuging for 10 mins at 1500 xg at room temperature and resuspended in PBS. Half of the washed HOU-treated platelet (PLT[t]) suspension was

added to an equal volume of the HOU-treated PPP (PPP[t]) and tested directly for platelet aggregation as above. Half of the washed untreated platelet (PLT[c]) suspension was added to an equal volume of the untreated PPP (PPP[c]) and tested directly for platelet aggregation. The second aliquot of washed HOU-treated platelets (PLT[t]) was added to untreated PPP (PPP[c]) and tested for platelet aggregation. The second aliquot of the washed untreated platelets (PLT[c]) was added to HOU-treated PPP (PPP[t]) and tested for platelet aggregation.

2.2.6 Platelet aggregation in HOU-treated plasma

In order to investigate whether the inhibition of platelet aggregation resulted from products of HOU-treated blood cells, the effect of HOU-treated plasma on the aggregation of untreated platelets was investigated.

Blood (40 ml) was collected from each of 4 individuals and divided into 2 x 10 ml and 1 x 20 ml aliquots.

One 10 ml aliquot acted as the control and the other 10 ml aliquot was treated with HOU (ozone concentration 15 μg / ml). They were tested for platelet aggregation as above.

The 20 ml aliqout was taken to prepare PRP by centrifugation at 200 xg for 15 mins at room temperature (18°C-25°C). The PRP was divided into two equal aliquots. These two aliquots of PRP were centrifuged to separate PPP and platelets. One PPP aliquot was treated with HOU (ozone concentration 15 μg / ml) and the other acted as the untreated control. One platelet aliquot was re-introduced to the PPP aliquot treated with HOU. The other platelet aliquot was added to untreated PPP as a control. They

were tested for platelet aggregation and the aggregation of the platelets in untreated control PPP was used as a control.

2.2.7 EDRF inhibitor - oxyhaemoglobin

The high affinity of nitric oxide for the Fe^{2+} moiety of oxyhaemoglobin was utilised to demonstrate whether the effect of the HOU-treatment of blood on platelet aggregation could be attributed to the released nitric oxide.

Oxyhaemoglobin reacts with NO to form a tightly bound complex:

$$NO + Hb_4O_8 \longrightarrow Hb_4[\,Fe^{2+}\,]O_8 \quad (\text{with } NO \text{ bound to } Fe^{2+})$$

Bovine haemoglobin (BHb) was dissolved in phosphate-buffered saline (PBS) to give a 1mM / L solution. As BHb contains both oxyhaemoglobin and a contaminating oxidized derivative methaemoglobin (metHb), the following purification procedure was employed. BHb was added to an 10M / L excess of the reducing agent sodium dithionite ($Na_2S_2O_4$). This was then purified by dialysis in a short length of Visking tubing immersed in distilled water for 2 hours at 4°C with stirring and the final product was stored at -20°C in 100 μl aliquots (Martin, et al, 1985).

Oxy-Hb 0.025 ml of a 10 μm / L solution was added to 0.225 ml PRP prior to the addition of platelet aggregatory agents. Platelet aggregation in the presence of this inhibitor of NO activity was compared with that in its absence.

2.2.8 Blood platelet and RBC counts and Hb measurement

Blood platelet counts were performed on whole blood (untreated control blood and HOU-treated blood), using a Coulter counter loaned by the Department of Haematology, Northern General Hospital, Sheffield, UK.

2.2.9 Nitrite and nitrate measurements

NO is unstable and decomposes rapidly in biological solutions to form mixtures of the stable derivatives, nitrite (NO_2) and nitrate (NO_3). Tolins and Shultz (1994) have demonstrated that serum and urine levels of NO_2 and NO_3 can be utilised as markers of endogenous NO activity.

PPP was prepared from either control or HOU-treated blood immediately after treatment by centrifugation at 1,500 xg and 4°C for 15 minutes and stored at -20 °C or below and the analysis was carried out as soon as possible.

The principle of the measurement of nitrite and nitrate is based on that fact the nitrate in a sample is reduced by a cadmium catalyst to nitrite, which reacts with the Griess reagent to form a purple azo dye. For the details of the preparation of the reagents and procedure see Appendix 2-3.

2.2.10 6-keto prostaglandin-F1-alpha measurements

2.2.10.1 Specimen collection and storage

Venous blood was collected from healthy volunteers and treated using the Ozon-O-Med device as above. Indomethacin was added to the blood immediately after treatment to

inhibit the subsequent metabolism of arachidonic acid to prostaglandins. PPP was prepared in the same way as for analysing NO and the plasma sample stored at -70°C until assay.

2.2.10.2 Extraction of 6-keto-prostaglandin F_{1a}

Solid phase extraction procedures result in a high recovery and clean plasma extract. In this experiment, Amprep C_2, 100 mg minicolumn were employed. For the method of extraction of 6-keto-prostaglandin F_{1a} see Appendix 2-4.

2.2.10.3 Radioimmunoassay

The concentration of 6-keto prostaglandin-F1-alpha in the extraxted sample was measured using a commercially available radioimmunoassasy kit. The 6-keto-prostaglandin-F1-alpha is the stable metabolite of prostacyclin and is a measure of prostacyclin concentration (Siess & Dray, 1982). For the assay principle and procedure see Appendix 2-5 and Appendix 2-6.

2.2.11 Platelet-derived growth factor (PDGF) measurement

20 ml venous blood from two healthy non-smoking volunteers was collected into EDTA. The blood was divided into two 10 ml aliquots. One aliquot was treated with HOU, the other was used as an untreated control. PRP was prepared from the control or treated blood, then 100 μl PBS, ADP (0.01 mM / L), collagen (0.1 mg / ml) or epirephrine (0.01 mM / L) were added to 900 μl of control PRP or treated PRP. After incubation for 1 hour to maximise the release of PDGF, the PRP containing PBS or platelet agonist were centrifuged at room temperature for 10 minutes at 1500 xg, the

PPP was removed from the sedimented platelets and the plasma stored at -70°C prior to analysis.

20 ml venous blood from six healthy non-smoking volunteers was collected into EDTA. The blood was divided into two 10 ml aliquots. One aliquot was treated with HOU, the other was used as an untreated control. PPP was prepared from control and treated blood by centrifugation at room temperature for 10 minutes at 1500 xg and stored at -70°C prior to analysis.

The concentration of PDGF in plasma was measured using a commercially available radioimmunoassay kit (Amersham International UK). The principle and procedure of the assay are similar to those of the commercial 6-keto-prostaglandin-F1-alpha assay. PDGF can be measured in the range 1.56 to 200 fmol / tube (46.8 - 6000 pg / tube).

II *In vitro* study in patients with PVD and diabetes

2.2.1 Patients

Fourteen patients with vascular disease only, 11 male, 3 female, aged between 50-60 years and 8 patients with diabetes, 5 male, 3 female, aged between 45-60 years were recruited for the study. These latter were a heterogeneous group and included patients with diabetes complicated by vascular disease and leg ulcer. All patients were from the in-patient and out-patient department of vascular disease at the Royal United Hospital, Bath.

The diagnosis of peripheral vascular disease was based on a typical history and clinical examination confirmed by angiography. All had limb pain at rest, limb oedema, change of skin pigmentation, desquamation, eczema and cyanosis; and some of them had skin

ulcers. The diagnosis of diabetes was also based on typical history and clinical examination confirmed by an increase in a level of glucose in blood and urine. Some of the diabetic patients were taking insulin or oral hypoglycaemic agents.

2.2.2 Treatment of blood samples

Blood (20 ml) was collected from each patient into tubes containing 3.8 % sodium citrate anticoagulant in a ratio of blood to anticoagulant of 9:1 and the 10 ml of the anticoagulated blood was placed into the reaction container of the Ozon-O-Med machine (Appendix 2-1). Blood was treated as described above, using an ozone concentration of 15 μg / ml in combination with a temperature of 42.5 °C and UV irradiation. The other 10 ml blood was used as an untreated control sample.

PRP and PPP from either the HOU-treated or control untreated sample were prepared for studying platelet aggregation and the measurement of 6-keto prostaglandin $F_{1\alpha}$ as described above.

2.2.3 Platelet aggregation in HOU-treated whole blood

Platelet aggregation was studied using the agonists ADP, thrombin and calcium ionophore A23187 in platelet rich plasma (PRP). Platelet aggregation in the treated samples was expressed as percentage of the aggregation in the control samples for each individual experimental condition.

2.2.4 6-keto-prostaglandin $F_{1\alpha}$ measurements

The method of extraction of 6-keto-prostaglandin F_{1a} are detailed in Appendix 2-4. The concentration of 6-keto-prostaglandin F_{1a} in plasma was measured using a commercially avaliable ELISA kit. For the priciple of the assay see Appendix 4-4.

III. Statistical analysis

Statistical comparisons were made using the Student's t test for paired samples, comparing treated sample with their corresponding controls. $P < 0.05$ (two tailed) was taken as significant. All values are expressed as the mean ± sd.

2.3. Results

I *In vitro* study on blood from healthy volunteers

2.3.1 Blood platelet, RBC counts and Hb measurement

Following treatment of 10 ml whole blood with 5-50 μg/ml of ozone in oxygen at an oxygen flow rate of 0.12 L / min for 3 mins at 42.5°C (total mass of ozone reacted 1.8 - 18.0 mg) and exposure to heat and UV light, there was an apparent overall increase in the platelet count to 186.5% ± 107.9% (range 65.5 - 461.5%, $P < 0.01$) of the control value in the 15 individuals investigated. This suggests that, under the conditions used in these experiments, the treatment of whole blood with heat, ozone / oxygen and ultraviolet irradiation does not destroy the blood platelets. A possible cause of this increase in platelet counts may be disaggregation of small platelet clumps present in the circulation by a de-aggregatory stimulus resulting from the blood treatment or from a blood sampling artifact.

Following treatment of 10 ml whole blood with 5-50 μg/ml of ozone in oxygen at an oxygen flow rate of 0.12 L / min for 3 mins at 42.5°C (total mass of ozone reacted 1.8 - 18.0 mg) and exposure to heat and UV light, there was no decrease in the RBC count (control 4.12 ± 0.81 x 10^{12} / L, treated blood 4.35 ± 0.54 x 10^{12} / L, n = 16) and no significant increase in the Hb (control 12.6 ± 2.07 g / dL, treated blood 13.2 ± 1.47 g / dL, n = 16). This suggests that, under the conditions used in these experiments, the treatment of whole blood does not cause a significant haemolysis compared with the control blood samples, indicating that the treatment regime had an insignificant effect on erythrocyte integrity.

2.3.2 Platelet aggregation in the plasma from HOU-treated whole blood

Platelets from blood treated with 5-50 μg / ml ozone as above showed a reduction in their ability to aggregate in response to ADP (0.001-0.05 mmol / L final concentrations, Figure 2-1). The overall inhibition of aggregation was 47.5% ± 28.6% (n=14, $P <$ 0.001). The inhibition was variable between individuals, ranging from 2.6 - 91%. There was no significant difference between the inhibitory effects on blood taken from males and females (inhibition of aggregation in males 40.8% ± 27.7%, n = 8, inhibition of aggregation in females 56.3% ± 29.8%, n = 6).

This inhibitory effect of HOU-treatment was dependent on the concentration of ADP between 0.001 mmol / L and 0.05 mmol / L, showing a higher level of inhibition of platelet aggregation at lower concentrations of ADP (Table 2-1). The inhibitory effect at final concentration 0.001 mmol / L ADP was significantly ($P < 0.05$) greater than at final concentration 0.05 mmol / L of this agonist (Table 2-1). However, this relationship was not observed at higher ADP concentrations.

Table 2-1. The inhibition of platelet aggregation in response to ADP by treatment of blood *in vitro* with HOU-treatment (ozone concentration 5-50 μg / ml).

ADP final concentration (mmol / L)	Inhibition of aggregation Mean (%)	Inhibition of aggregation Sd (%)	Number of observations	P*
0.001	65.3	23.4	8	0.001
0.005	53.3	29.4	5	0.02
0.01	42.7	31.9	10	0.01
0.05	32.0**	29.1	8	0.02

* - compared to the untreated control sample.

** - significantly different from 0.001 mmol/L ADP, $P < 0.05$.

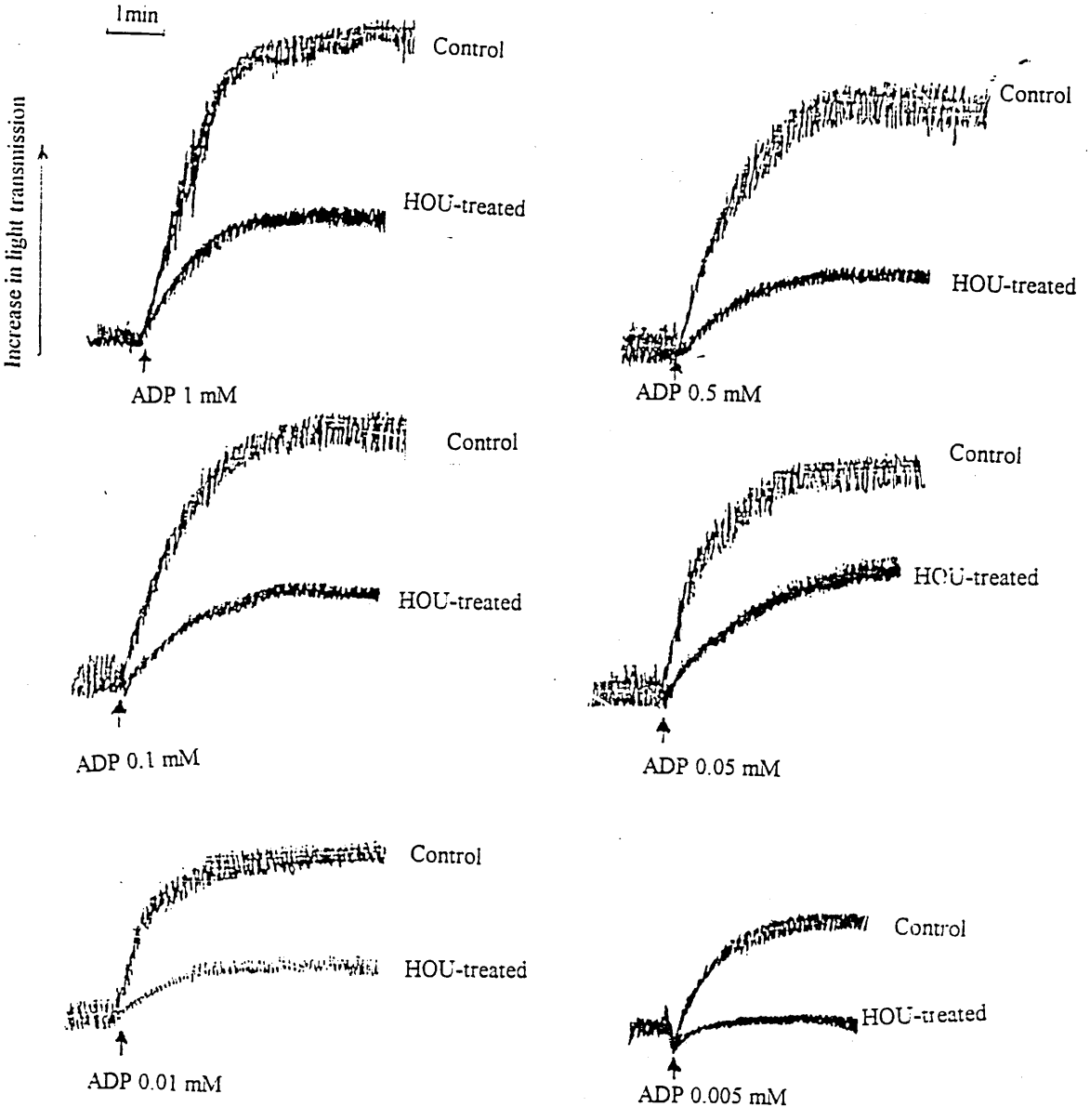


Figure 2-1. Platelet aggregation induced by different concentrations of ADP in untreated control and HOU-treated blood obtained from a normal, healthy volunteer. The inhibition of the platelet aggregation in the blood treated with HOU is dependent on the concentration of ADP, greater inhibition was displayed at lower ADP concentrations (concentration of ozone in oxygen: 25 μg / mL).

Whole blood treated with 5 - 35 μg / ml ozone in the presence of heat and UV also showed a high level of inhibition of platelet aggregation using collagen as the platelet agonist (Table 2-2).

Whole blood treated with 5 - 40 μg / ml ozone, heat and UV showed a higher level of inhibition of aggregation (up to 94.2% ± 5.3%, $P < 0.001$, n = 5) using the calcium ionophore A23187 as platelet agonist (Table 2-3).

With thrombin as the inducer of platelet aggregation, platelets from whole blood treated with 5 - 40 μg / ml ozone, heat and UV also showed an inhibition of aggregation (Table 2-4).

A reduction in the concentration of ozone in the oxygen bubbled through the blood sample resulted in a reduction in the effect of treatment on the inhibition of platelet aggregation in two of the four individuals investigated. This difference was significant in the responses of the same individual to the treatment, although the overall mean values of the four individuals investigated were not significantly different (Table 2-5) (Figure 2-2).

Table 2-2. The inhibition of platelet aggregation in response to collagen by HOU-treatment (ozone 5 - 35 μg / ml).

Collagen final concentration (mg / mL)	Inhibition of aggregation Mean (%)	Inhibition of aggregation Sd (%)	Number of observations	P*
0.02	79.2	24.9	4	0.01
0.1	66.7	37.5	5	0.02
1.0	73.4	16.3	4	0.01

* - compared to the untreated control sample.

Table 2-3. The inhibition of platelet aggregation in response to calcium ionophore A23187 by HOU-treatment (ozone 5 - 40 μg / ml).

Calcium ionophore A23187 final concentration (μg/mL)	Inhibition of aggregation Mean (%)	Inhibition of aggregation Sd (%)	Number of observations	P*
0.5	60.7	35.8	5	0.02
1.0	43.1	47.8	6	NS
5.0	94.2	5.3	5	0.001
10.0	62.6	36.9	5	0.02

* - compared to the untreated control sample.

NS - no significant difference.

Table 2-4. Inhibition of platelet aggregation in response to thrombin by HOU-treatment (ozone 5 - 40 μg / ml).

Thrombin final concentration (u / mL)	Inhibition of aggregation Mean (%)	Inhibition of aggregation Sd (%)	Number of Observations	P*
0.1	52.7	37.1	5	0.05
0.5	68.2	17.7	6	0.001

* - compared to the untreated control sample.

Table 2-5. The comparison of the inhibition of platelet aggregation by ADP in blood HOU-treatment in different concentrations of ozone.

SUBJECT	5 μg / ml ozone	25 μg / ml ozone	50 μg / ml ozone
Male 1	15.5	53.9	65.6 *
Male 2	8.7	11.2	24.7 **
Female 1	87.3	91.6	93.9
Female 2	42.3	69.2	39.4
Mean (n=4)	38.5	56.6	55.9
Sd	35.7	33.9	30.5

* - significant different from ozone 5 ug / ml at $P < 0.01$

** - significant different from ozone 5 ug / ml at $P < 0.02$

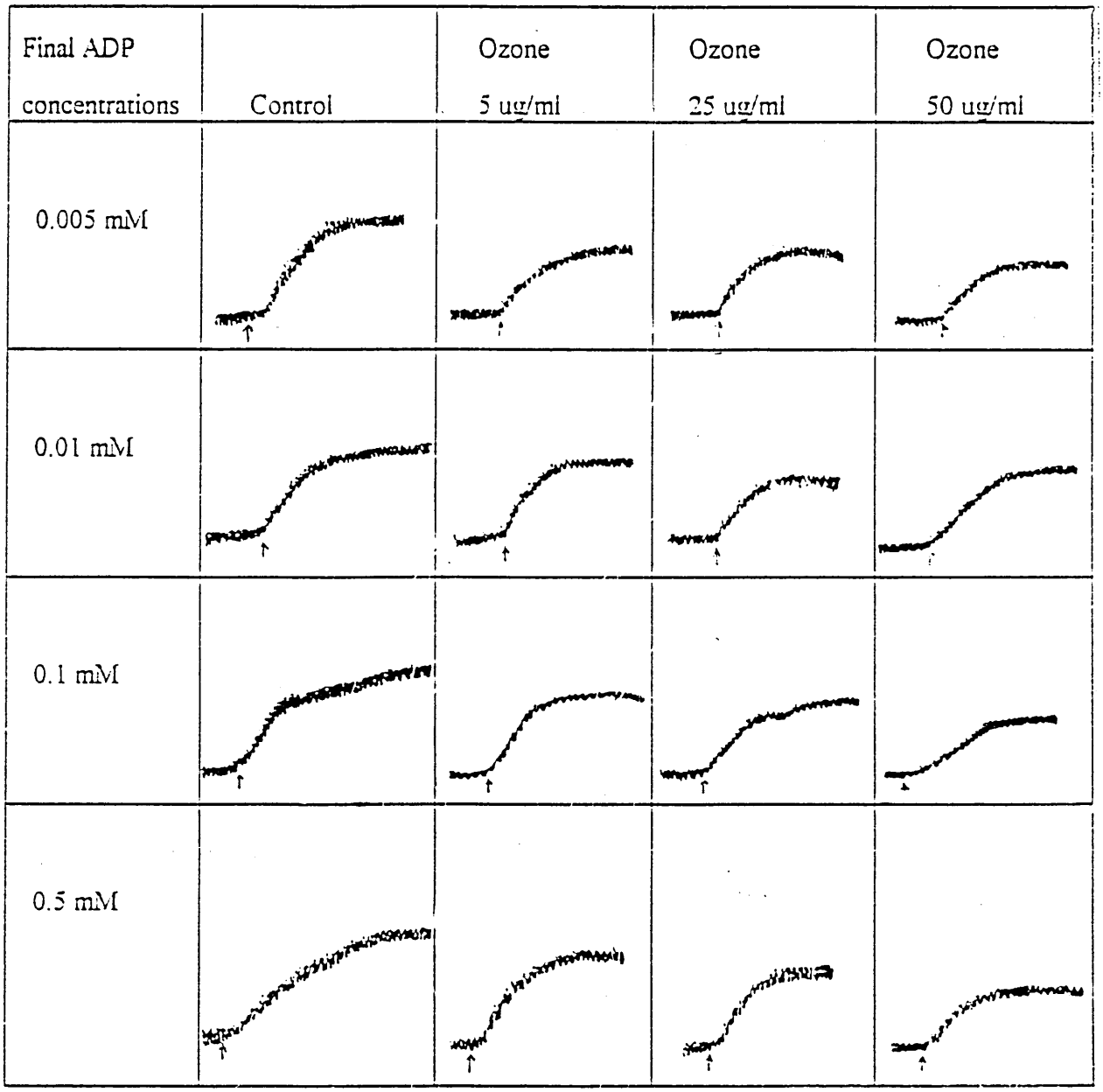
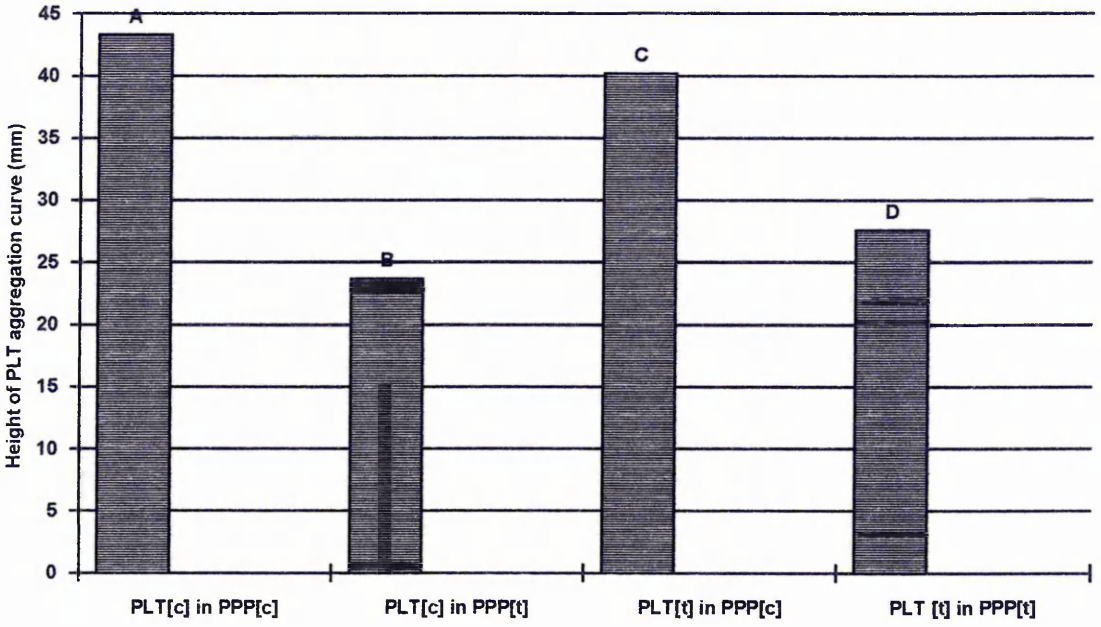


Figure 2-2. Platelet aggregation after blood treated with different ozone concentration 5, 25, 50 μg / ml in the present of heat and UV.

2.3.3 The aggregation of washed platelets in the plasma from HOU-treated whole blood

The washed untreated control platelets (PLT[c]) were added to the washed HOU-treated PPP (PPP[t]) (ozone 15 μg/ml) and the untreated control PPP (PPP[c]) respectively. There was a reduction of PLT[c] aggregation ability in the PPP[t] compared to in the PPP[c] using ADP, thrombin and calcium ionophore A23187 as the platelet agonists (for the concentrations of these three platelet agonist see Appendix 2-6). The height of the aggregation curve of PLT [c] in PPP [c] was 43.3 mm ± 6.2 mm, and the height of the aggregation curve of PLT [c] in PPP [t] was 23.7 mm ± 13.8 mm, $P < 0.02$, n = 5 (Figure 2-3 A, B). For the platelet aggregation of the 5 individuals see Appendix 2-6.

The washed HOU-treated platelets (PLT[t]) (ozone 15 μg/ml) were added to the PPP[c] and the PPP[t] respectively. The aggregation ability of the PLT[t] showed a reduction in the PPP[t] compared to in the PPP[c] using ADP, thrombin and calcium ionophore A23187 as the platelet agonists (for the concentrations of these three platelet agonist see Appendix 2-6). The height of the aggregation curve of PLT [t] in PPP [c] was 40.2 mm ± 11.7 mm, and the height of the aggregation curve of PLT [t] in PPP [t] was 27.6 mm ± 12.3 mm, $P < 0.05$, n = 5 (Figure 2-3 C, D). For the platelet aggregation of 5 individuls see Appendix 2-6. There were no significant difference either between A and C or between B and D.



PLT[c] - washed untreated control platelets

PLT[t] - washed HOU-treated platelets

PPP[c] - untreated control plasma

PPP[t] -HOU-treated plasma.

Figure 2-3. Comparation of aggregation of washed platelet from control and HOU-treated blood induced by the platelet agonists.

$P < 0.02$, when A compared with B.

$P < 0.05$, when C compared with D.

2.3.4 Platelet aggregation in HOU-treated plasma

Table 2-6 shows the comparison between HOU-treatment of whole blood and HOU-treatment of plasma in the inhibition of platelet aggregation. All four individuals demonstrated significant inhibitions of platelet aggregation in HOU-treated whole blood, $P < 0.05$, 0.01, 0.05 and 0.02 respectably. Treatment of plasma with HOU, by contrast, did not result in the significant inhibition of platelet aggregation.

Table 2-6. Comparison of the inhibition (%) of platelet aggregation in the HOU-treated blood and in the HOU-treated plasma.

Subjects	1		2		3		4	
Samples	Treatment of whole blood	Treatment of plasma	Treatment of whole blood	Treatment of plasma	Treatment of whole blood	Treatment of plasma	Treatment of whole blood	Treatment of plasma
Inhibition mean (%)	55.8	38.8	50.8	26.5	59.2	8.4	56.4	2.4
Sd (%)	20.9	34.1	26.6	31.5	41.6	17.1	37.1	6.4
Numbers of experiment	7	7	9	9	12	12	7	7
P*	< 0.05	NS**	< 0.01	NS	< 0.05	NS	< 0.02	NS

* *P* were calculated in comparison to the control samples for each individual experimental condition.

** NS No significant difference.

2.3.5 The effect of oxyhaemoglobin on inhibition of platelet aggregation

Oxyhaemoglobin was added after the blood was HOU-treated and before aggregation was investigated. The inhibitory effect of platelet aggregation by HOU-treatment decreased when the oxyhaemoglobin was added to PRP from HOU-treated blood compared to the equivalent volume of phosphate-buffered saline (0.025 ml) to the PRP (Figure 2-4). The inhibition of platelet aggregation in response to ADP without oxy-Hb was 63.1% ± 36.9%, and 34.5% ± 38.7% with oxy-Hb, $P < 0.05$, n = 4. The inhibition of platelet aggregation in response to thrombin without oxy-Hb was 64.5% ± 32.9%, and 41.4% ± 39.1% with Oxy-Hb, $P < 0.05$, n = 6 (Figure 2-5).

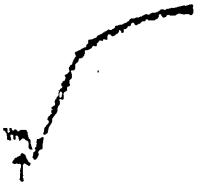
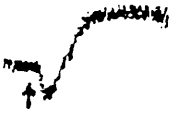
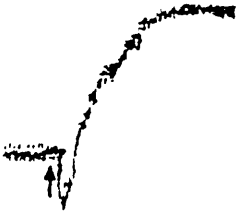
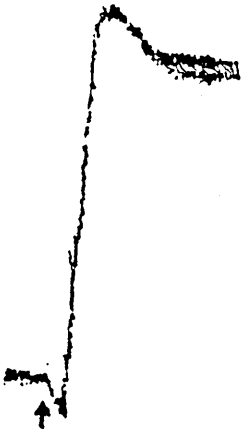
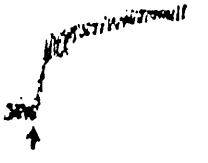
Final platelet aggregatory reagents	Control	Ozone 43 ug/ml	Ozone 43 ug/ml with Oxy-Hb
ADP 0.01mM			
Thrombin 1u			
Thrombin 10u			

Figure 2-4. platelet aggregation after blood treated with HOU in the present and absent of oxyhaemoglobin.

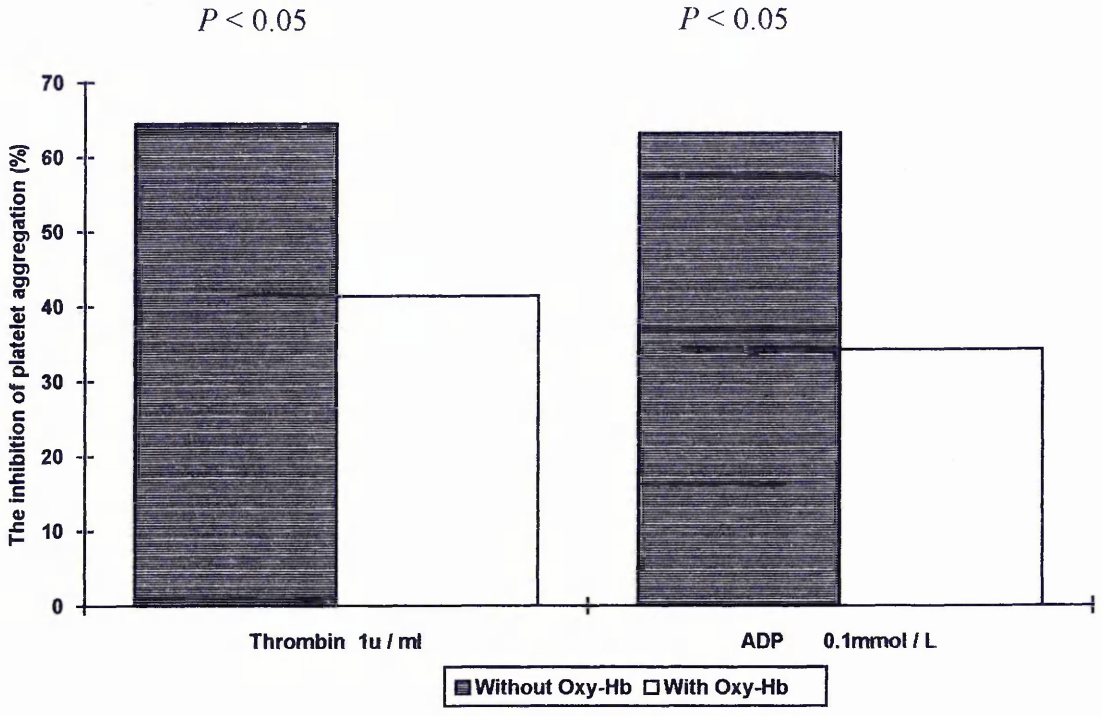


Figure 2-5. The reversal by oxyhaemoglobin of the inhibition of platelet aggregation following treatment of blood with oxygen / ozone, heat and UV irradiation.

2.3.6 Nitrite and nitrate measurement

Ozone, heat and UV treatment of whole blood resulted in an increase in the concentration of nitrite plus nitrate (69.6 μM ± 36.9 μM) compared to the corresponding untreated control sample (51.15 μM ± 27.1 μM), $P < 0.05$, n = 10 (Figure 2-6). When plasma was treated with Ozon-O-Med rather than the blood containing the cellular components, no increase in nitrite could be detected, suggesting that the increase in nitrite is derived from blood cells.

2.3.7 6-keto-$PGF_{1\alpha}$ measurement

The concentration of 6-keto prostaglandin-$F_{1\alpha}$ was 47.4 pg / ml ± 15.6 pg / ml in the post-treatment samples and 24.5 pg / ml ± 9.30 pg / ml in the pre-treatment samples (n = 12). This difference was significant at $P < 0.001$ (Figure 2-7).

The concentration of 6-keto-PGF_{1a} following treatment of blood with UV light only was not significantly different from the pre-treatment sample (14.30 pg / ml ± 3.9 pg / ml with UV, control 12.9 ± 1.6, n = 4)

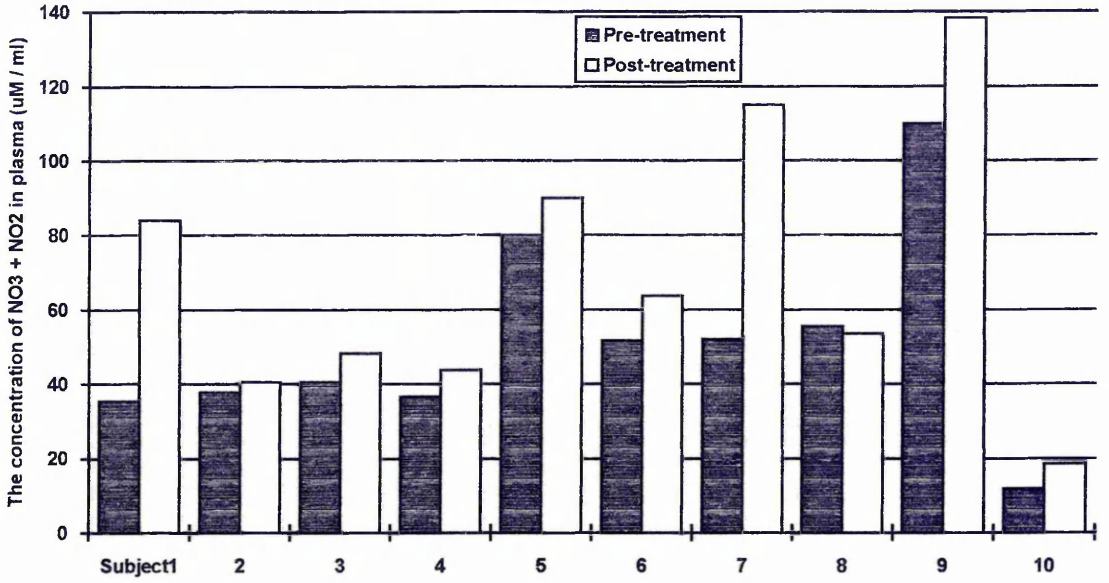


Figure 2-6. The changes of the concentration of nitrate plus nitrite in plasma.

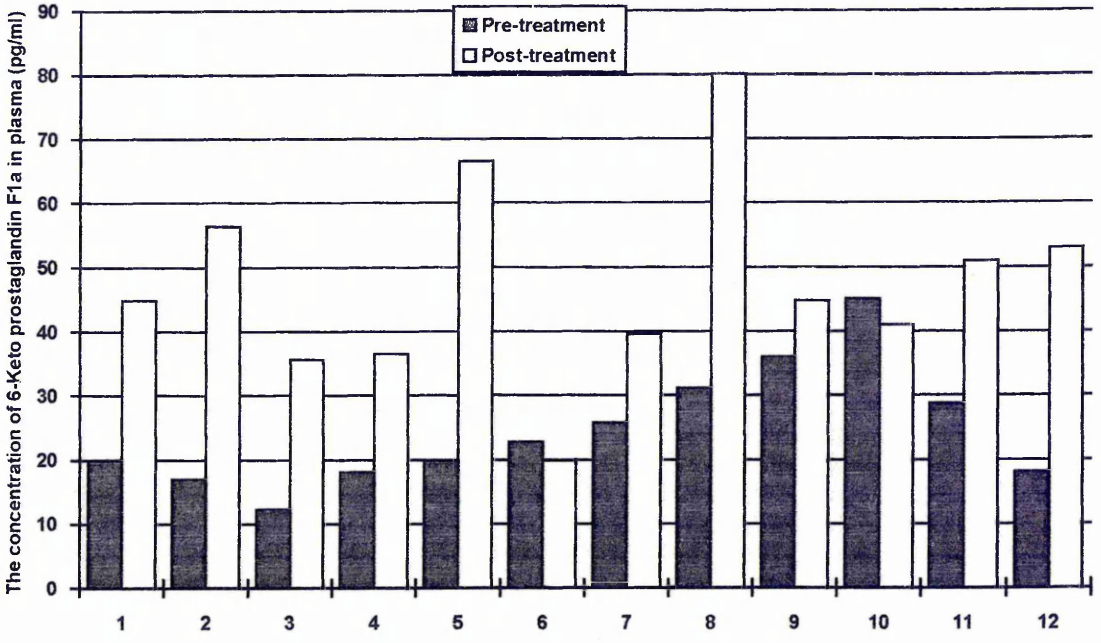


Figure 2-7. The changes of the concentration of 6-keto prostaglandin-$F_{1\alpha}$ in plasma following treatment of whole blood with HOU.

2.3.8 Platelet-derived growth factor (PDGF)

Table 2-7 shows the concentrations of PDGF in plasma after PRP from untreated control and HOU-treated whole blood was exposed to platelet aggregating agents. In this experiment, the amount of PDGF released by platelets from treated whole blood did not show any significant difference compared with the control sample.

Table 2-8 showed concentrations of PDGF in plasma from control untreated and treated whole blood. The levels of PDGF in the treated whole blood did not show any significant difference compared with the control sample.

Table 2-7. Comparison of release of PDGF in control and HOU-treated blood sample in response to platelet agonists (fmol / tube).

	Control				Ozone 50 μg/ml			
Platelet aggregating reagent	PBS	ADP 0.01mM/L	Collagen 0.1 mg/ml	Epinephrine 0.01 mM /L	PBS	ADP 0.01 mM/L	Collagen 0.1 mg/ml	Epinephrine 0.01 mM /L
Subject 1	6.1	16.4	9.6	13.5	7.3	14.1	12.8	6.8
Subject 2	10.3	11.3	8.4	8.4	6.4	5.8	7.4	13.2
Mean (n = 2)	8.20	13.85	9.00	10.95	6.85	9.95	10.10	10.00

Table 2-8. Comparison of the concentrations of PDGF in control and HOU-treated blood samples (ozone 50 μg/ ml) (fmol / tube).

Subject No	Control	Treatment
1	14.30	9.27
2	11.20	34.63
3	15.86	49.53
4	15.57	48.63
5	6.1	7.30
6	10.3	6.40
Mean (n = 6)	12.22	25.96
Sd	3.77	20.75

II *In vitro* study in patients with PVD and diabetes

2.3.1 Platelet aggregation

After 10 ml of whole blood was treated with 15 μg / ml ozone in the presence of heat and UV irradiation, either in the patients with vascular disease or in the patients with diabetes, platelets showed a reduction in their ability to aggregate in response to ADP, thrombin and calcium ionophore A 23187 (Table 2-9 and Table 2-10).

In blood collected from the patients with vascular disease, after treatment with HOU *in vitro*, there was a significant inhibition of platelet aggregation in response to calcium ionophore A 23187 at final concentrations of 10 μg / mL and 5.0 μg / mL, 77.3 % ± 32.4 % (n = 12, $P < 0.001$) and 56.0 % ± 39.4 % (n = 12, $P < 0.001$) respectively, when compared to the control samples. With thrombin at final concentrations of 1 u / mL and 0.5 u / mL as platelet agonist, the inhibition of aggregation was 79.3 % ± 27.0 % (n = 10, $P < 0.001$) and 83.8 % ± 18.1 % (n = 8, $P < 0.001$) respectively when compared to the control samples. With final a concentration of 10 μM / L ADP as the inducer of platelet aggregation, the inhibition of aggregation showed a higher level 94.0 % ± 9.4 % (n = 6, $P < 0.001$) when compared to the control samples (Table 2-9).

Table 2-9. Inhibition of platelet aggregation *in vitro* in the vascular disease patients group after *ex vivo* treatment of blood using HOU.

Platelet agonist and final concentration	Calcium-ionophore-A 23187		Thrombin		ADP
	10 μg /ml	5 μg /ml	1 u / ml	0.5 u / ml	10 μM / L
Mean of inhibition of platelet aggregation (%)	77.3	56.0	79.3	83.8	94.0
Sd (%)	32.4	39.4	27.0	18.1	9.4
n	12	12	10	8	6
P*	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

* - comparing with the corresponding untreated control sample.

In blood collected from the diabetic patients, after blood was treated with HOU *in vitro*, there was a significant inhibition of platelet aggregation in response to calcium ionophore A 23187 at final concentrations of 10 μg /ml and 5.0 μg / ml, 55.9 % ± 30.9 % (n = 8, $P < 0.002$) and 68.8 % ± 35.6 % (n = 7, $P < 0.01$) respectively, when compared to the control samples. With thrombin at final concentrations of 1 u / mL and 0.1 u / mL as agonist, the inhibition of aggregation was 50.8 % ± 33.2 % (n = 8, $P < 0.01$) and 47.9 % ± 37.3 % (n = 8, $P < 0.01$) respectively, when compared to the control samples. With final a concentration of 10 μM / L ADP as the inducer of platelet aggregation, the inhibition of aggregation was 43.6 % ± 30.4 % (n = 6, $P < 0.02$) when compared to the control samples (Table 2-10).

Neither in the patients with vascular disease nor the diabetic patients, were there significant differences in the inhibition of platelet aggregation between the two different final concentrations (10 μg / mL and 5 μg / mL) of calcium-ionophore-A 23187 investigated. Likewise, there were also no significant differences in the inhibition of platelet aggregation at the two different final concentrations 1 u / mL and 0.5 u / mL of thrombin used.

Table 2-10. Inhibition of platelet aggregation *in vitro* in the group of diabetic patients after *ex vivo* treatment of blood using HOU.

Platelet agonist and final concentration	Calcium-ionophore-A 23187		Thrombin		ADP
	10 μg /ml	5 μg /ml	1 u	0.5 u	10 μM / L
Mean of inhibition of platelet aggregation (%)	55.9	68.8	50.8	47.9	43.6
Sd (%)	30.9	35.6	33.2	37.3	30.4
n	8	7	8	8	6
P*	< 0.002	< 0.01	< 0.01	< 0.01	< 0.02

* - comparing with untreated control sample.

2.3.2 6-keto-prostaglandin F_{1a}

Table 2-11 shows that 6-keto-prostaglandin F_{1a} levels in the plasma of 14 patients with vascular disease, measured by an enzyme-linked immunosorbent assay, were 42.76 ± 24.6 pg /mL in control untreated samples and 119.82 ± 99.46 pg / ml after HOU-treatment. This increase was significant ($P < 0.02$).

Table 2-12 shows that in the eight diabetic patients, the concentration of 6-keto-prostaglandin F_{1a} in the plasma was 27.75 ± 9.99 pg /mL in the control untreated samples and 53 ± 18.38 pg / mL after treatment samples . This increase was significant ($P < 0.05$).

Table 2-11. The concentration of 6-keto-PGF_{1a} (pg / ml) in blood plasma from patients with vascular disease before and after the *ex vivo* treatment of whole blood using HOU.

Patients	Control sample	Treated sample
1	39.36	233.0
2	48.3	361.3
3	22.56	266.6
4	108.3	137.6
5	11.7	34.5
6	23	57
7	30	94
8	29	120
9	52	31
10	56	70
11	44	43.5
12	19.5	41
13	67	96
14	48	92
Mean	42.76	119.82 *
Sd	24.6	99.46

* $P < 0.02$ compared to the control

Table 2-12. The concentration of 6-keto-PGF_{1a} (pg / ml) in blood plasma from patients with diabetes before and after the *ex vivo* treatment of whole blood using HOU.

Patients	Control sample	Treated sample
1	16.5	62.0
2	30.5	45.0
3	38.0	33.0
4	44.0	49.0
5	26.0	49.0
6	15.0	44.0
7	29.5	48.0
8	22.5	94.0
Mean	27.75	53.0 *
Sd	10.0	18.38

* $P < 0.05$ compared to the control

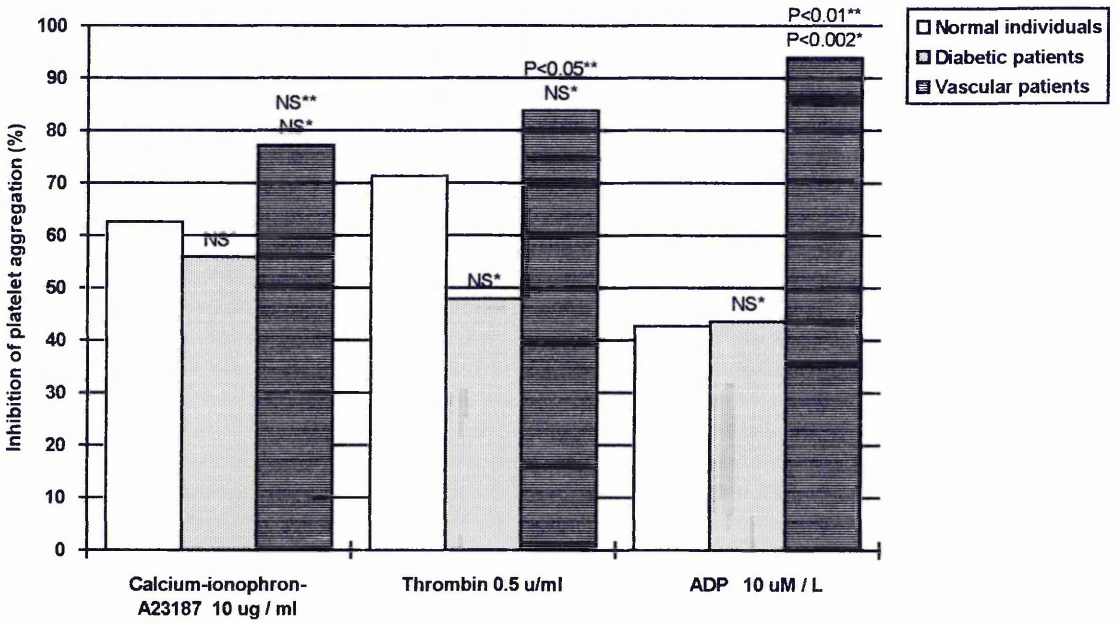
III. Comparisons of the inhibition of platelet aggregation and the levels of 6-keto-prostaglandin $F_{1\alpha}$ in the healthy volunteers and patients with PVD and diabetes

Results from normal healthy volunteers were compared with these from the two patient groups. However, this comparison was not totally appropriate as they were of a lower age range (20-50 years) than the patients.

Figure 2-8 shows the comparisons of inhibition of platelet aggregation *in vitro* in three groups (normal individuals, vascular disease and diabetic patients) after HOU-treatment of blood with the Ozon-O-Med device. A significantly greater inhibition of platelet aggregation was apparent in the vascular disease patients group compared to the normal controls when 10 μmol / L ADP was used as the platelet agonist ($P < 0.002$). When the vascular disease patient group was compared with the diabetic patient group, significantly greater inhibition of platelet aggregation were apparent in the vascular disease patient group when using 0.5 u /mL thrombin ($P < 0.05$) and 10 μmol / L ADP ($P < 0.01$) as platelet agonists. Platelet from the diabetic and the healthy volunteers showed no difference in their response to *ex vivo* HOU treatment.

Figure 2-9 shows the comparisons of 6-keto-prostaglandin $F_{1\alpha}$ concentrations before and after *in vitro* blood HOU-treatment in normal, vascular disease patients and diabetics. The levels of 6-keto-prostaglandin $F_{1\alpha}$ were significantly higher in treated vascular patients' blood than in treated blood from diabetics or the healthy volunteers ($P < 0.05$). The levels of 6-keto-prostaglandin $F_{1\alpha}$ in untreated blood is not significantly different in these three groups.

Figure 2-8. Comparisons in the inhibition of platelet aggregation *in vitro* after *ex vivo* HOU-treatment of blood from normals, vascular disease patients and diabetics



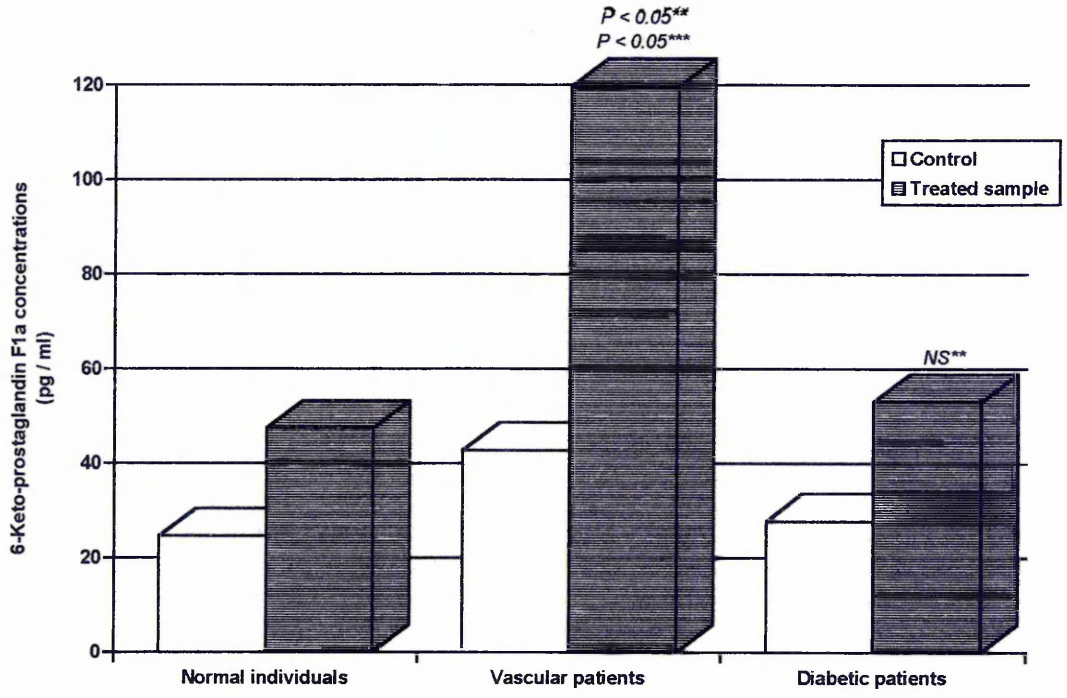
n = 5 8 12 6 8 8 10 6 6

* - compared to normal individuals;

** - compared to the diabetic patients.

NS - no significant difference.

Figure 2-9. Comparisons of 6-keto-prostaglandin $F_{1\alpha}$ concentrations before and after *in vitro* blood HOU-treatment in normal, vascular disease patients and diabetics.



*P**	< 0.001	< 0.02	< 0.05
n =	12	14	8

* control sample comparing with the corresponding treated sample.

** comparing with normal individuals' HOU-treated samples.

*** comparing with diabetic patients' HOU-treated samples.

NS no significant difference $P > 0.05$.

2-4 Discussion

There are three basic pathways of platelet aggregation: the release of ADP, the liberation and metabolism of arachidonate, and the synthesis of platelet-aggregating factor (PAF), all of which are interlinked. ADP causes a chain of events involving phosphatidyl inositol metabolism, leading to the exposure of fibrinogen binding sites on the platelet membrane and aggregation. Collagen causes platelet aggregation through a pathway dependent on prostaglandin biosynthesis. This induces arachidonic acid mobilisation from the membrane, followed by conversion to thromboxane A_2, which is a potent stimulator of platelet aggregation, causing ADP release and calcium flux. Similar considerations apply to thrombin. Adrenaline acts synergistically with other reagents (The British Society for Haematology BCSH Haemostasis & Thrombosis Task Force, 1988). A third pathway of platelet activation has been suggested to be the formation of PAF, although its site of synthesis and catabolism and its mechanism of stimulation of platelets are unknown. PAF aggregates rabbit platelets in the subnanomolar concentration range, and human platelets at 10 times higher concentrations. Rabbit platelets also form larger amounts of PAF upon stimulation by thrombin, although the formation of PAF by human platelets is less easily detected (Longenecker, 1985). Each one of these pathways generates compounds which act as intercellular mediators to propagate the rapid activation of all the platelets. It is currently thought that the mechanism of stimulation by endogenous pathways is through a common intercellular trigger, possibly calcium (Longenecker, 1985).

The experimental results of these *in vitro* studies showed that platelet aggregation was inhibited when whole blood was treated with heat, ozone / oxygen and ultraviolet using the Ozon-O-Med device. The inhibition of the platelet aggregation response shows a decrease in the height and the slope of the aggregation curve. The inhibition of platelet aggregation might result from the direct effect of HOU-treatment on platelets or by the

production of inhibitors of platelet aggregation produced by the HOU-treatment of whole blood cells or plasma.

A higher concentration of ADP (0.05 mmol / L) resulted in a lower inhibitory effect (32 % ± 29.1 %) on platelet aggregation than lower concentrations of ADP (0.001 mmol / L, inhibition 65.3 % ± 23.4 %, n = 8, $P < 0.05$), which suggests that this observed inhibition is reversible (Table 2-1). This reversible inhibition may be explained as the higher agonist concentrations partially overcoming the inhibitory effect of heat, oxygen / ozone and ultraviolet by hyperstimulating the platelets. That this inhibition is reversible indicates that the function of platelet aggregation is not damaged by HOU-treatment. The results from studies using washed platelets also indicated that the observed inhibition of platelet aggregation by HOU-treatment was not due to direct platelet damage - platelets isolated from treated blood maintained their ability to aggregate in the absence of plasma from treated blood.

The inhibition of aggregation of treated platelets also occurs in response to collagen, thrombin and the calcium ionophore A 23187. These results suggest that the inhibition of platelet aggregetion by HOU-treatment may act via a decrease in the calcium flux into platelets.

HOU-treatment of blood resulted in an apparent increase in total blood platelet count ($P < 0.01$). This is probably due to disaggregation of small platelet clumps present in the circulation by the de-aggregatory stimulus resulting from the blood treatment.

The results from the studies on washed platelet aggregation indicated that some inhibitors in the plasma are derived from treated whole blood. A comparison of platelet aggregation in the plasma from HOU-treated whole blood with plasma treated directly with HOU demonstrated a significant inhibition of platelet aggregation in HOU-treated

whole blood but not in HOU-treated plasma. This suggests that the inhibitors may be produced by the blood cells and not directly by the effects of either heat, UV light or ozone on plasma.

Nitric oxide plays a role in the inhibition of platelet adhesion and aggregation by the stimulation of soluble guanylate cyclase and inducing a rise in intracellular levels of cyclic GMP in platelets (Synder & Bredt, 1992). Nitric oxide also acts upon vascular smooth muscle via a similar pathway causing relaxation and vasodilation.

In the HOU-treated whole blood there was a significant increase in the concentration of nitric oxide, as measured by its stable metabolites nitrite plus nitrate. Evidence that the inhibition of the platelet aggregation may be partly caused by NO increase is indicated by the reversal by oxyhaemoglobin in the inhibition of platelet aggregation. Oxyhaemoglobin inhibits NO by chemically binding (Synder & Bredt, 1992; Salvimini et al, 1990; Ignarro [1], 1989; Sarrel, et al, 1990). NO is a small and lipophilic molecule. It rapidly permeates the biological membrane barrier. Oxyhemoglobin cannot penetrate platelets, so the reversing effect must be due to removal of NO from the extracellular medium to create a concentration gradient which then results in a reduced NO concentration inside the platelets (Ignarro, 1990).

Prostacyclin inhibits platelet aggregation and induces vasodilatation by stimulating adenylate cyclase and inducing an elevation in intracellular levels of cyclic AMP in vascular smooth muscles and platelets (Moncada & Vane, 1979; Vane & Botting, 1995). cAMP is thought to regulate calcium levels by promoting its uptake into the dense tubular system. cGMP regulates calcium levels by inhibiting calcium influx and mobilization from intracellular stores. Therefore the final common result is a reduction in calcium availability (Radomski et al, 1990).

Monocytes produce small amounts of PGI_2 by the cyclooxygenase pathway and IL-1 can enhance monocyte PGI_2 synthesis (Hakkert, et al, 1992). The results (Figure 2-7) showed that there was an increase in the concentrations of prostacyclin, as measured by its stable metabolites 6-keto $PGF_{1\alpha}$, in the HOU-treated blood. Furthermore, higher levels of 6-keto $PGF_{1\alpha}$ were found in plasma prepared from HOU-treated blood of vascular disease patients (Figure 2-9) - correlating with an increased platelet inhibiting effect of treatment on blood from these patients (Figure 2-8). The increase in prostacyclin may be explained that UV, ozone/oxygen and IL-1 released by monocytes and neutrophils from HOU-treated blood, induce the synthesis of cycloxygenase by trigging NF-kB in monocytes.

PDGF was originally identified as a product of platelets and is a specific protein of platelets. It is present in the alpha-granules and represents the major mitogenic constituent of serum (Heldin, et al 1985, Ross, et al 1986). It is released from platelets upon exposure to ADP, thrombin and collagen and may be part of the repair response to thrombolytic injury (Ross, et al 1986). Measurement of the blood platelet alpha-granule component platelet-derived growth factor in plasma from HOU-treated and untreated blood revealed no significant difference, indicating that this treatment did not affect the platelet release reaction or disrupt the integrity of the platelet.

The results from the *in vitro* studies on the vascular and diabetic patients also show the inhibition of platelet aggregation in response to ADP, thrombin and calcium ionophore A23187 and the significant increases in prostacyclin levels, as measured by its stable metabolites 6-keto $PGF_{1\alpha}$, after blood treated with HOU.

In summary, these *in vitro* results suggest that HOU-treatment of whole blood using the Ozon-O-Med device can inhibit platelet aggregation. This treatment does not damage platelets. The inhibition of platelet aggregation is probably due to the increases in the

synthesis of prostacyclin and nitric oxide by leucocytes activated by HOU. These observations provide a basis supporting the empirical observation of improvements in peripheral vascular disease following Ozon-O-Med and similar ozone-based treatments.

CHAPTER 3

Safety of Minor Autohaemotherapy with Blood Treated with Heat, Oxygen/Ozone and UV Irradiation

3.1 Introduction

Any new drug or device needs to have proven safety and efficacy before translating into a medical therapy. There are a number of ways of checking safety, the most basic approach is by the observations of clinical symptoms, signs and objective laboratory results from a clinical trial involving a small number of volunteers treated with the new drug or device.

For over 3 decades approximately 380,000 patients had been treated with ozonized blood autohaemotherapy (Jacobs, 1982) or HOU-autohaemotherapy in Western Germany. However, the application of ozone therapy and HOU-therapy remains unproved because of insufficient objective laboratory results for judging adverse effects.

Objectives

The aim of this study was to assess the effect of HOU-minor autohaemotherapy, in which 10 ml of blood is treated with oxygen containing ozone at a concentration of 15 μ g / ml at an elevated temperature (42.5°C) and in the presence UV irradiation, using the 'Ozon-O-Med' device and then injected to the donor intramuscularly

The following investigations were performed on healthy volunteers after a course of HOU-treatment.

(a) Side effects on clinical examination

(b) Standard haematological parameters in peripheral venous blood: haemoglobin (Hb), white blood cell (WBC) count, differential WBC, platelet count, red blood cell (RBC) count, RBC indices (Hct - haematocrit, MCV - mean corpuscular volume, MCH - mean corpuscular haemoglobin).

(c) Standard clinical biochemistry parameters in peripheral venous blood: urea, sodium (Na^{+}), potassium (K^{+}), bicarbonate, creatinine, total protein, albumin, bilirubin, alkaline phosphatase (Alk. phos), gamma-glutamyltransferase (Gamma G. T.), aspartate transaminase (AST) and lactate dehydrogenase (LDH).

(d) Urinary dipstick tests.

3.2 Methods

3.2.1 Subjects

19 healthy, non-smoking volunteers aged 20-30 years were recruited in the study. 15 volunteers were male and 4 were female. All were judged to be healthy on the basis of a medical history, physical examination, urinary dipstick tests and clinical biochemistry results (urea, Na^{+}, K^{+}, bicarbonate, creatinine, total protein, albumin, bilirubin, Alk. phos, Gamma G. T., AST and LD) and haematology results (Hb, WBC count, differential, platelet count, RBC count and RBC indices, which includ Hct, MCV, MCH).

Five of the 19 subjects were allocated to a control group. These 5 male volunteers were

given a sham treatment by intramuscular re-injection of their own untreated blood. The treated volunteers (10 male, 4 female) were re-injected with their own blood after exposure to the heat, oxygen / ozone and UV irradiation stimuli. Subjects were randomly allocated to the control or treatment group and treated in a double blind fashion, i.e. neither the volunteer, the clinician administering the treatment, nor the individual carrying out the laboratory studies were aware of what treatment was used in any particular case.

The subject No13. in the HOU-treated group showed a blood urea of 7.5 mmol / L which is outside the quoted normal range prior to the HOU-treatment, but clinically, there was no apparent cause for this increased value.

Volunteers were asked to avoid drugs known to affect platelet function (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988), including: aspirin, beta-blockers, antihistamines, tricyclic antidepressants, NSAIDs, corticosteroids and antibiotics, through the study and for two weeks beforehand.

The study was approved by the medical research ethics committee of Bassetlaw Hospital and Community Health Service (Barrowby House, Highland Grove, Worksop, Nottinghamshire, United Kingdom, S 81 0JN) and all subjects gave their written informed consent to participate in the study.

3.2.2 Exclusions

The contraindications for ozone therapy include acute alcohol intoxication, recent myocardial infarction, haemorrhage, hyperthyroidism and thrombocytopenia (Rilling & Viebahn, 1987). Individual with a previous history of anaphylaxis were excluded, as

were pregnant or lactating females.

3.2.3 Trial design and statistical analysis

By taking blood samples prior to the minor autohaemotherapy treatment regime, each of the individuals acted as their own control. Before / after treatment changes were measured (see Table 3-1). Results were expressed as mean ± sd. Statistical evaluation was performed using the student's t test for paired samples with $P < 0.05$ as the minimum level of significance.

Table 3-1. Proocal for autohaemotherapy and blood sampling

			Blood tested for:		
Day	bleed vol	re-inject vol	biochemistry	haematology	activity of immune cell mediators (PGI_2, NO, IFN-gamma and IL-2)*
Mon	40 ml	10 ml	10 ml	10 ml	10 ml
Wed	20 ml	10 ml			10 ml
Fri	20 ml	10 ml			10 ml
Mon	20 ml	10 ml			10 ml
Wed	10 ml	10 ml			
Wed(+2h)**	30 ml		10 ml	10 ml	10 ml
Fri	10 ml				10 ml
1 week later	10 ml				10 ml

* Details given in Chapter 4.

**30 ml blood were collected 2 hours after the injection of HOU-treated or sham-treated blood

3.2.4 Methodology

The reagents and equipment used are specified in Appendix 4-4.

Blood (10 ml) was collected into sodium citrate anticoagulant, pharmaceutical grade for injection (from Quarzlampenfabrik, Dr Muller, Gmbh, Essen, Germany) (2.0 ml of a 3.13 % solution, final blood citrate concentration 0.018 mol/L) and 10 ml of the anticoagulated blood was placed into the reaction vessel of the Ozon-O-Med equipment (Appendix 2-1). After connecting the thermocouple and gas inlet tube to the machine, a standard three minutes treatment cycle was initiated, using a pre-set ozone concentration of 15 μg / ml. The medical grade oxygen was converted to ozone by silent electrical discharge at ambient temperature, and the mixture of oxygen and ozone was then bubbled through the sample for 3 min. The temperature of the blood was raised to 42.5° C and the blood was also exposed to UVC irradiation, at a wavelength of 253.7 nm and a dose of 6.0 mJ / cm^2, during this treatment. After treatment, the blood was withdrawn into a syringe, then mixed with 2 ml of a local anaesthetic procaine (1%), pharmaceutical grade (from Quarzlampenfabrik, Dr Muller, Gmbh, Essen, Germany) and injected intramuscularly into the gluteal muscle (Appendix 2-2).

A disposable sterile pack is provided for each treatment, containing a syringe, sodium citrate, reaction vessel, gas inlet tube and thermocouple (Appendix 2-2). Each sterile pack is used once only, to exclude cross-contamination of blood during treatment in the Ozon-O-Med equipment.

This procedure was carried out 5 times over a 2 week period. Control subjects had blood withdrawn, anticoagulated as above but the blood was not treated with ultraviolet light, heat and oxygen / ozone. The untreated blood was reinjected intramuscularly after

mixing with 2 ml procaine (1%).

3.2.5 Clinical monitoring of individuals

All individuals were kept under observation for 60 minutes after each treatment as a precaution against an anaphylactic reaction. Blood pressure was taken immediately before and 1 hour after each minor autohaemotherapy treatment.

Any adverse events noted at the time of treatment or reported later by any individual were recorded and, if necessary, further evaluated and followed up.

3.2.6 Urine test

Before each treatment and at the second day after the final treatment, urine was tested for blood, bilirubin, protein (albumin is indicated with greater sensitivity than other proteins), ketones, ascorbic acid, glucose and pH using a Maxi Screen Combi 6A (from Cambrige Selfcare Diagnostics limited, UK) test strip with vitamin C alert.

3.2.7 Laboratory studies

A standard haematology screen (Hb, WBC count, differential, platelet count, RBC count and RBC indices, which included Hct, MCV, MCH) and clinical biochemistry profile (urea, Na^+, K^+, bicarbonate, creatinine, total protein, albumin, bilirubin, Alk. phos, Gamma G. T., AST and LD) was carried out before and after the course of treatment at the Department of Haematology and Clinical Chemistry at the Victoria Hospital, Worksop.

3.3 Results

3.3.1 Clinical monitoring

All 19 subjects (14 HOU-treated and 5 control subjects) were monitored within 1 hour of receiving each treatment for their immediate general reaction. In neither the control nor the HOU-treated groups after injection did any individual complain of specific symptoms such as fever, headache and malaise. There was no swelling, redness or tenderness in the local areas of the injection. There were no acute allergic reactions. After treatment, the following symptoms were investigated: pruritis, change of taste, fever, buccal ulceration, purpura, epitaxis, bleeding (e.g. gum), bruising, diarrhoea, uricaria, eczema, dyspnea and chest pain. No individual complaimed of any of the above. Blood pressure and pulse were normal before and 1 hour after each minor autohaemotherapy treatment. There were no significant changes in blood pressure or pulse rate either in the control or the HOU-treated groups (Table 3-2 and 3-3).

3.3.2 Urine test

No abnormalities were observed.

Table 3-2. Changes in the blood presure and pulse of pre-treatment and post-final treatment in the HOU treated group.

	Pre-treatment		Post-final treatment	
Subject No	Blood presure (systolic/diastolic)	Pulse (/ min)	Blood presure (systolic/diastolic)	Pulse (/ min)
1	93 / 65	56	112 / 70	57
2	120 / 80	100	100 / 70	82
3	110 / 70	60	130 / 70	60
4	119 / 93	61	103 / 63	59
5	80 / 70	46	80 / 60	50
6	101 / 57	61	120 / 70	65
7	127 / 91	100	116 / 90	75
8	133 / 87	87	111 / 61	76
9	122 / 70	58	149 / 57	57
10	125 / 80	64	123 / 73	48
11	120 / 80	62	100 / 70	82
12	100 / 70	69	122 / 90	84
13	130 / 80	70	127 / 76	70
14	133 / 71	73	117 / 69	82
mean*	115 / 76	69	115 / 71	68
sd	16 / 10	16	16 / 10	13

* No significant changes in blood pressure or pulse rate.

Table 3-3. Changes in the blood presure and pulse of pre-treatment and post-final treatment in the control group.

	Pre-treatment		Post-final treatment	
Subject No	Blood presure (systolic/diastolic)	Pulse (/ min)	Blood presure (systolic/diastolic)	Pulse (/ min)
1	129 / 100	62	109 / 91	55
2	123 / 75	53	126 / 85	61
3	122 / 77	50	119 / 65	62
4	118 / 73	61	112 / 66	47
5	120 / 69	60	110 / 70	60
Mean*	122 / 79	57	115 / 75	57
Sd	4 / 12	5	7 / 12	6

* No significant changes in blood pressure or pulse rate.

3.3.3 Haematology screen

Table 3-4 and Table 3-5 summarise the results of measurements of haematological parameters in the HOU-treated and control groups. All parameters were within the quoted normal range for all individuals both before and after treatment (see Appendix 3-1 for comprehensive data).

There was a significant increase in lymphocyte counts after HOU-treatment in treated group ($P < 0.01$, $n = 14$), but still all within quoted normal range. There was no significant difference in lymphocyte counts when compared to the control group. No significant changes were observed in the other parameters.

3.3.4 Biochemistry screen

Table 3-6 and Table 3-7 summarise the results of measurements of clinical biochemistry profiles in the control and HOU-treated group. Appendix 3-2 show the results of clinical biochemistry screen for all individuals (in the HOU-treated and control group) both before and after treatment. One subject (No13) in the HOU-treated group, had a pre-treatment blood urea concentration (7.5 mmol / L) above normal. This returned to within normal limits (5.4 mmol / L) following treatment. Except for this individual, all results were within the quoted normal range for all individuals both before and after treatment.

In conclusion, there was no evidence that the HOU treatment in this small group was associated with any adverse reactions or any abnormality in biological function.

Table 3-4. Haematological parameters in HOU-treated group (n = 14)

Haematological parameter		Pre-treatment Mean	Sd	After-treatment Mean	Sd	Normal range
Haemoglobin	g / dl	14.42	1.10	13.99	0.97	F 11.5 - 16.5 M 13.0 - 18.0
White blood cells	10^9 / L	6.15	1.66	6.65	1.70	4.0 - 12.0
Platelet	10^9 / L	258.85	45.61	252.14	38.06	150 - 400
Hct	L / L	0.417	0.032	0.406	0.029	F 0.37 - 0.47 M 0.49 - 0.54
Red blood cells	10^{12} / L	4.78	0.42	4.64	0.34	F 3.8 - 5.8 M 4.5 - 6.5
MCV	fl (femtoliters)	87.42	2.91	87.52	3.52	78 - 98
MHC	pg	30.22	1.33	30.17	1.41	27 - 32
Neutrophils	10^9 / L	3.42	1.59	3.83	1.63	2.0 - 7.5
Eosinophils	10^9 / L	0.21	0.14	0.20	0.14	0.1 - 0.4
Basophils	10^9 / L	0.038	0.05	0.025	0.046	0 - 0.1
Lymphocytes	10^9 / L	1.88	0.49	2.10*	0.46	1.5 - 4.0
Monocytes	10^9 / L	0.61	0.24	0.52	0.21	0.2 - 0.8

* $P < 0.01$ compared to pre-treatment.

Hct Hematocrit

MCV Mean Corpuscular Volume

MCH Mean Corpuscular Hemoglobin

Table 3-5. Haematological parameters in control untreated group (n = 5)

Haematological parameter		Pre-treatment Mean	Sd	After-treatment Mean	Sd	Normal range
Haemoglobin	g / dl	14.80	0.62	14.38	0.52	F 11.5 - 16.5 M 13.0 - 18.0
White blood cells	10^9 / L	6.80	1.45	7.28	1.25	4.0 - 12.0
Platelet	10^9 / L	271.40	30.94	261.20	43.57	150 - 400
Hct	L / L	0.429	0.013	0.410	0.015	F 0.37 - 0.47 M 0.49 - 0.54
Red blood cells	10^{12} / L	4.78	0.16	4.48	0.21	F 3.8 - 5.8 M 4.5 - 6.5
MCV	fl (femtoliters)	90.84	1.90	91.46	2.65	78 - 98
MCH	pg	31.34	0.63	32.06	1.14	27 - 32
Neutrophils	10^9 / L	3.64	1.25	4.10	1.04	2.0 - 7.5
Eosinophils	10^9 / L	0.30	0.18	0.32	0.14	0.1 - 0.4
Basophils	10^9 / L	0.02	0.04	0.08	0.08	0 - 0.1
Lymphocytes	10^9 / L	2.26	0.32	2.20	0.30	1.5 - 4.0
Monocytes	10^9 / L	0.6	0.27	0.58	0.14	0.2 - 0.8

Hct Hematocrit

MCV Mean Corpuscular Volume

MCH Mean Corpuscular Hemoglobin

Table 3-6. Biochemistry parameters in HOU treated group (n = 14)

Biochemistry parameter		Pre-treatment Mean	Sd	After-treatment Mean	Sd	Normal range
Urea	mmol / L	4.49	1.22	4.70	0.95	2.5 - 6.5
Sodium	mmol / L	140.71	1.26	141.78	1.25	135 - 145
Potassium	mmol / L	4.10	0.20	4.20	0.37	3.5 - 5.0
Bicarbonate	mmol / L	29.14	3.46	30.92	3.19	22 - 32
Creatinine	umol / L	87.71	8.51	95.25	7.68	45 - 120
Total Protein	g / L	74.64	4.81	73.38	2.75	60 - 88
Albumin	g / L	46.92	2.23	46.69	2.28	35 - 50
Bilirubin	g / L	12.64	5.58	12.61	7.61	3 - 21
Alk.Phos	u / L	184.42	51.91	170.23	44.60	80 - 300
Gamma G. T.	u / L	15	9.55	13.30	7.02	7 - 50
AST	u / L	19.14	4.80	17.46	4.29	0 - 40
LD	u / L	285.78	35.41	280	39.39	230 - 460

Alk.Phos Alkaline phosphase

Gamma G. T. Gamma-Glutamyltransferase

AST Aspartate transaminase

LD Lactate Dehydrogenase

Table 3-7. Biochemistry parameters in control untreated group (n = 5)

Biochemistry parameter		Pre-treatment Mean	Sd	After-treatment Mean	Sd	Normal range
Urea	mmol / L	4.80	1.11	4.32	0.66	2.5 - 6.5
Sodium	mmol / L	140.20	1.09	142.60	2.70	135 - 145
Potassium	mmol / L	4.12	0.30	4.38	0.19	3.5 - 5.0
Bicarbonate	mmol / L	29.00	3.53	32.40	3.50	22 - 32
Creatinine	umol / L	85.33	5.13	95.00	14.67	45 - 120
Total Protein	g / L	72.60	3.36	71.00	3.00	60 - 88
Albumin	g / L	46.80	1.92	46.40	1.14	35 - 50
Bilirubin	g / L	11.80	5.35	8.20	4.26	3 - 21
Alk.Phos	u / L	161.80	34.16	151.00	26.59	80 - 300
Gamma G. T.	u / L	23.80	19.38	21.60	16.56	7 - 50
AST	u / L	16.60	3.43	16.20	2.58	0 - 40
LD	u / L	282.60	55.13	267.60	41.64	230 - 460

Alk.Phos Alkaline phosphase

Gamma G. T. Gamma-Glutamyltransferase

AST Aspartate transaminase

LD Lactate Dehydrogenase

3-4 Discussion

All haematological and biochemical parameters were in normal ranges, suggesting that HOU-treatment with ozone 15 μg/ml does not induce a hepatic or renal dysfunction .

The results of this investigation support currently published data on the safety of HOU-autohaemotherapy (Viehausen, 1988; Garber et al, 1991).

In 1988, Dr Med. Gunther Viehausen observed 50 subjects aged 50-60 years treated with Ozon-O-Med equipment. His procedure was carried out 6 times over 3 weeks. Clinical and laboratory chemical examinations were carried out prior to and six weeks after treatment. One case of chronic progressive hepatitis and six cases of diabetes mellitus were diagnosed during the pre-treatment assessment of these subjects. The other 43 were normal healthy subjects. Subjectively, patients reported that they felt better during treatment, and that afterwards they felt stronger and slept better. No adverse effects were detected in tests of target organ system, such as the liver and kidney. In some cases abnormal laboratory parameters prior to HOU-therapy, normalised or showed reducing abnormalities after Ozon-O-Med treatment.

Garber et al (1991) performed a pilot study of minor autohaemotherapy with HOU-treated blood in 18 patients with HIV. The treatment was carried out three times per week for 8 weeks followed by 4-weeks observational follow-up. No significant bruising occurred and there were no incidences of abscess or other localised complications. All haematological and biochemical parameters were stable. The treatment did not cause haemolysis, hepatic or renal dysfunction in these patients. Again, the treatment was well tolerated.

Bocci and his colleagues were unable to find any toxic effects of ozonized blood autohaemotherapy in a recent study (1994) of 4 normal volunteers.

These data clearly show that HOU treated blood used intramuscularly is remarkably free of adverse effects, extending the small amount of published information.

CHAPTER 4

The Effects of Minor Autohaemotherapy with Blood Treated with Heat, Oxygen/Ozone and UV Irradiation

4.1 Introduction

Endothelial dysfunction plays a critical role in peripheral vascular disease (Vane, et al, 1990; Rau, 1991; Ware & Heistad, 1993; Ross, 1993). Many *in vitro* and *in vivo* experiments have demonstrated that a deficiency in the release of nitric oxide and prostacyclin by endothelial cells contributes to vascular disease (Radomski, et al, 1987 [3]; Willis, et al, 1986; Vallance, et al, 1989; Panza, et al, 1990; Tolins & Shultz, 1994; Chester, et al, 1990).

Initial studies *in vitro* (Chapter 2) have demonstrated that, following whole blood treated with a mixture of oxygen and ozone, heat and UV irradiation, there is a decrease in platelet aggregation to various agonists and evidence of an increase in the levels of prostacyclin and nitric oxide. While these findings *in vitro* provide some scientific validity for the anecdotal claims of efficacy in peripheral vascular disease following Ozon-O-Med and similar ozone-based treatments, it does not explain how the administration of a small volume of treated blood (10mL) can have the profound systemic activity suggested by these clinical observations. In order to attempt to explain this, further work on minor autohaemotherapy with the HOU-treated blood was performed.

The claimed benefits for ozone-therapy have already been outlined in Chapter 1. The therapeutic benefit in various fungal, viral diseases and tumours appears unlikely to be due to a direct viricidal properties of ozone, because ozone and peroxides decompose

very rapidly and the small (10 ml) blood sample only represents a minimal fraction of the internal milieu. Many studies have discovered that, *in vitro*, appropriate concentrations of ozone can activate monocytes and lymphocytes, and induce the release of an array of cytokines such as IL-1β, IL-2, IL-6, TNFα, IFNβ, IFNγ and GM-CSF (Bocci & Paulesu, 1990; Paulesu, et al, 1991; Bocci, et al, 1994). With these discoveries *in vitro*, a reasonable hypothesis has been proposed: ozone may act mainly via the stimulation of monocytes and lymphocytes as an inducer of release of cytokines (IFNs, LIs, TNFα, CSF). These cytokines in turn may stimulate an array of immune functions such as activation of macrophages and neutrophils, enhanced expression of major histocompatibility complex class I and II antigens on infected cells, monocytes and lymphocytes, thus leading to an activation of the host's immune system (Bocci, 1992; 1994). If this hypotheses was shown to be true, it could provided a rational basis for understanding the mechanisms of action of autohaemotherapy with blood exposed to ozone *ex vivo* in peripheral vascular disease, viral diseases and neoplasms.

Objectives

The aims of this study were to assess the effects of minor autohaemotherapy with blood treated with heat, oxygen / ozone and UV irradiation on:

(a) The concentrations of certain mediators of platelet aggregation in the plasma prepared from peripheral venous blood from treated volunteers.

(b) The activation status of peripheral blood immune cells from treated volunteers.

(c) The cytokine levels in the plasma prepared from peripheral venous blood from treated volunteers.

4.2 Materials and methods

The reagents and equipment used are listed in Appendix 4-3-1 and 4-3-2.

4.2.1 Subjects

This study was performed on the nineteen subjects as already mentioned in Chapter 3. They were healthy, non-smoking volunteers within the age range 20-30 years of age. For two weeks beforehand and during the study the subjects did not taken any drugs. Of the 19 individuals, a control group of 5 individuals (male) were given a sham treatment by intramuscular re-injection of their own untreated blood. The treatment group of 14 individuals (10 male, 4 female) were re-injected with their own blood after exposure to the heat, oxygen / ozone and UV irradiation stimuli. For treatment protocol see Table 3-1 in Chapter 3.

4.2.2 Samples

Blood samples were collected at intervals throughout the study as indicated in the protocol given in Table 3-1. In order to assess the expressions of certain molecules which are characteristic of activated mononuclear cells, peripheral mononuclear cells (PBMCs) were isolated and slides were prepared for microscopy using alkaline phosphatase/anti-alkaline phosphatase (APAAP) immunoenzymatic staining. Plasma was prepared for measurements of nitrite plus nitrate, 6-keto-$PGF_{1a,}$, IFNγ and IL-2 .

1 year after the minor autohaemotherapy, the concentration of 6-keto-PGF_{1a} was measured in plasma samples from HOU-treated or the sham treated subjects.

4.2.3 Preparation of mononuclear cells

Mononuclear cells were isolated from whole blood by density gradient centrifugation on Histopaque 1077 (Appendix 4-1) and were washed 3 times with DMEM. The cell pellet was then resuspended in DMEM (1 x 10^6 cells / ml) and 10 μl aliquots was placed in individual wells of a multispot slide and left to air dry. Slides were stored at -20 °C in aluminium foil until required.

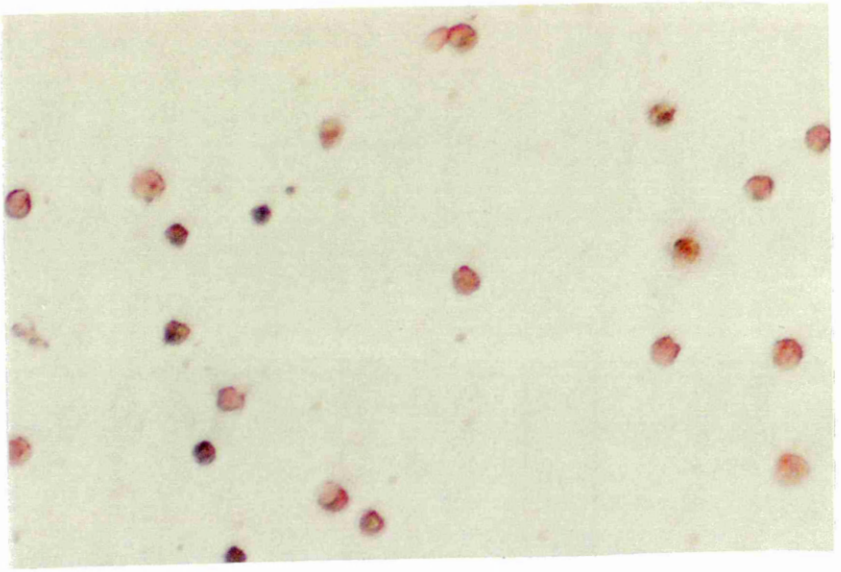
4.2.4 Alkaline phosphatase/anti-alkaline phosphatase (APAAP)

APAAP immunoenzymatic staining method was employed to assess the expression of certain molecules on PBMCs. For the priciple of APAAP immuno-enzymatic staining see Appendix 4-2-1. The APAAP immuno-enzymatic staining procedure is described in detail in Appendix 4-2-2. The monoclonal mouse antibodies used in this study are shown in Table 4-1. The percentage of positive expression of each specific antigen on PBMCs was calculated from the ratio of the number of positive cells to the number of PBMC (CD45 positive cells) per high power field (see Figure 4-1).

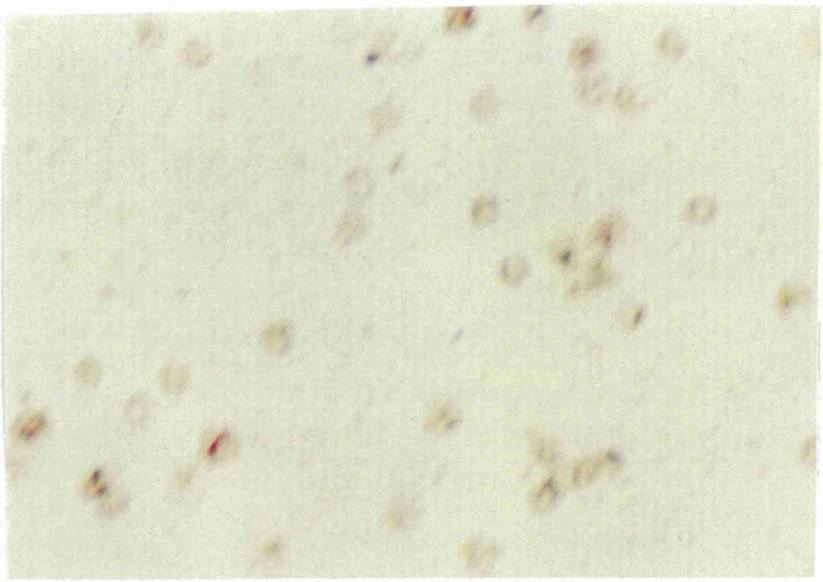
Table 4-1. Monoclonal mouse antibodies detected at leucocyte antigens.

MAb	(Antigen)	Source	Specificity
HLe-1	(CD45)	Becton-Dickinson Oxford, UK.	Reacts strongly with all leucocytes.
HLA-DR	(CR3/43)	Dako, Denmark.	Identifies HLA-DR molecules of the major histocompatibility complex (MHC class II) activated macrophage / monocytes and some B cells.
IL-2R	(CD25)	Dako, Denmark.	Identifies interleukin-2 (ACT-1) receptor on activated T lymphocytes.
Macrophage	(Ber-Mac3)	Dako, Denmark.	Identifies activated monocytes.

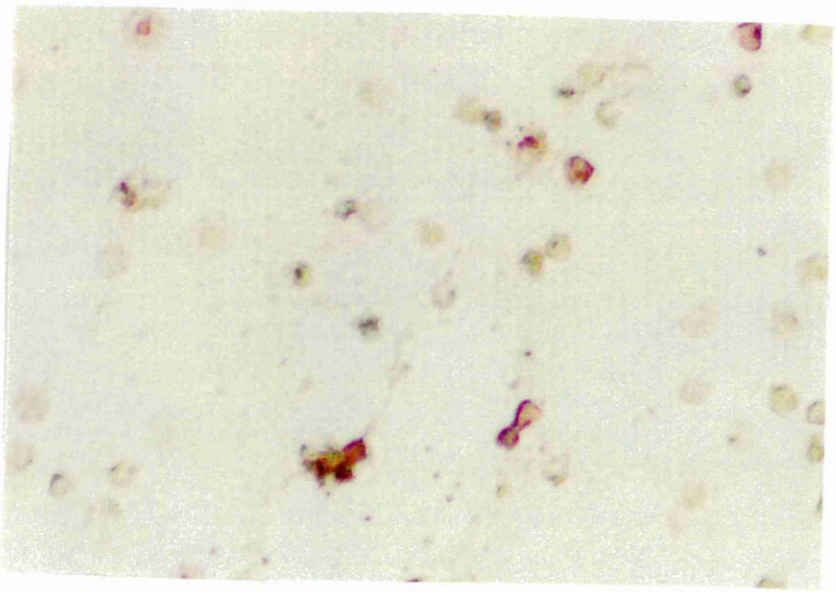
Figure 4-1. Photomicrographs of APAAP stained peripheral blood mononuclear cells (magnification = x1000)



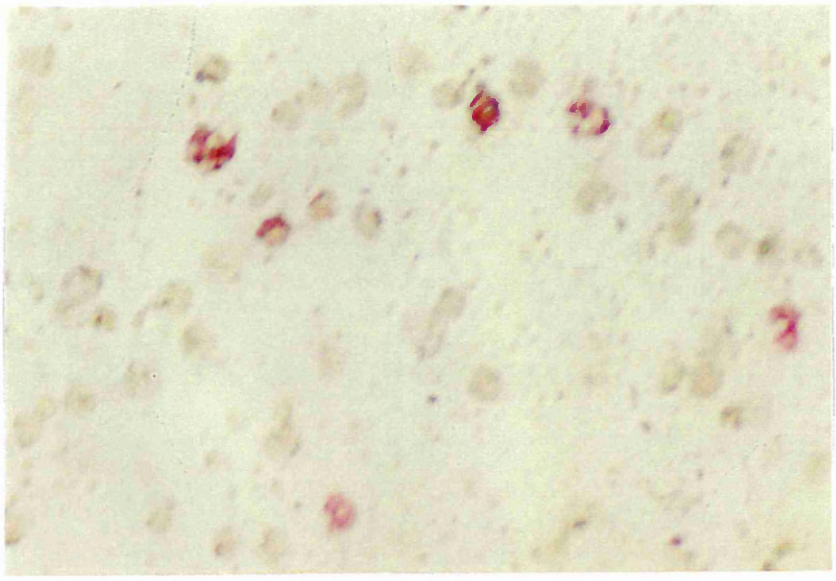
(a)Peripheral blood mononuclear cells (PBMCs) stained by CD45



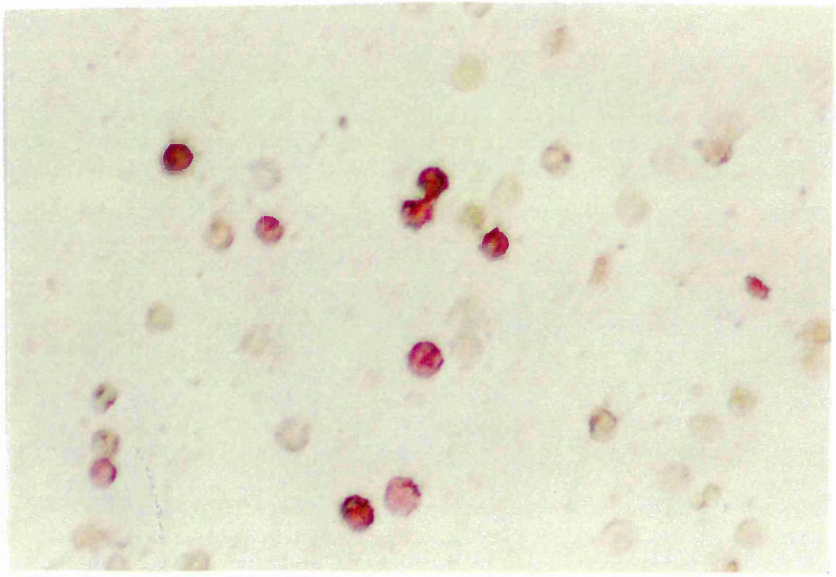
(b) CD25 staining activated T lymphocytes in PBMCs before HOU-autohaemotherapy



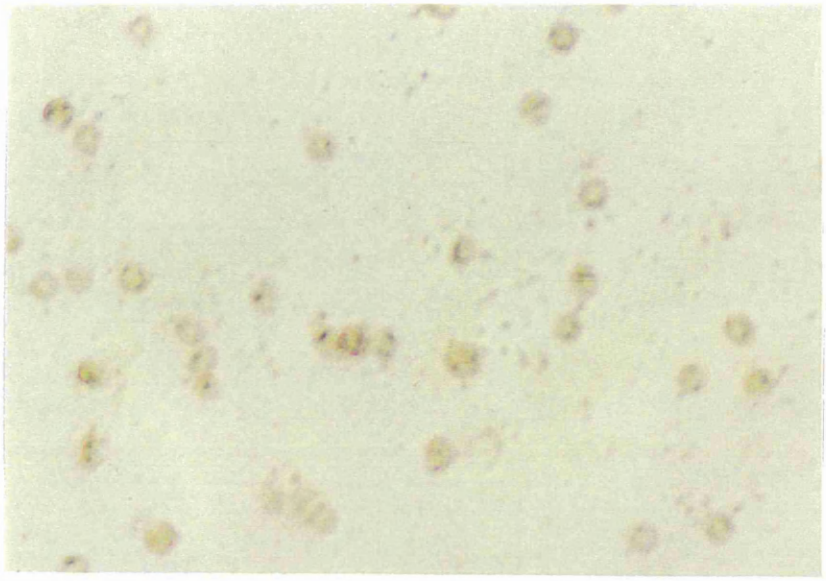
(c) CD25 staining activated T lymphocytes in PBMCs after HOU-autohaemotherapy



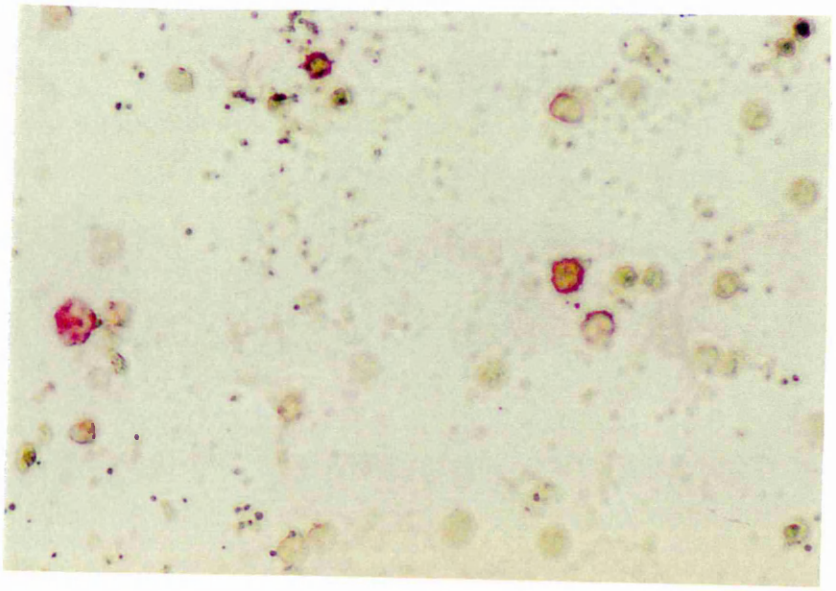
(d) HLA-DR staining activated mononuclear cells in PBMCs before HOU-autohaemotherapy



(e) HLA-DR staining activated mononuclear cells in PBMCs after HOU-autohaemotherapy



(f) Ber-Mac3 staining activated monocytes in peripheral blood before HOU-autohaemotherapy



(g) Ber-Mac3 staining activated monocytes in peripheral blood after HOU-autohaemotherapy

4.2.5 Nitrite and nitrate measurement

The preparation of plasma, the method of reduction from nitrate to nitrite and the measurement of nitrite using the Griess reagent are described at Appendix 2-3.

4.2.6 EIA for 6-keto-prostaglandin F1a

The sample preparation and method of extraction of 6-keto-prostaglandin F1a are detailed in Chapter 2 and Appendix 2-4. The concentration of 6-Keto-prostaglandin-F1-alpha in plasma was measured using a commercially avaliable ELISA kit. For the priciple of the assay see Appendix 4-3.

4.2.7 ELISA assay for IFN-gamma and IL-2

The concentration of IFN-gamma in plasma was measured using a commercially available ELISA kit (Endogen Inc, 451D street, Boston, MA, USA). This kits detects a minimum level of 5 pg / mL of biologically active interferon-gamma. Expected IFN-gamma values in most healthy individuals are less than 10 pg / mL.

The levels of IL-2 in plasma was also measured by an ELISA method (T Cell Diagnostic, Inc, 38 Sidney Street, Cambridge, MA02139)

4.2.8 Statistical analysis

Results are expressed as mean ± sd. The paired student's t-test was used to compare pre and post-treatment values. A P value of = or < 0.05 was taken as significant.

4.3 Results

4.3.1 Expression of molecules (surface markers) on PBMCs

4.3.1.1 Expression of CD25 on PBMCs

Table 4-2 shows the expression of CD25 measured by the APAAP method on the PBMCs of healthy volunteers following a course of autohaemotherapy with HOU-treated homologous blood. There was a gradually increase in the proportion of PBMCs expressing CD25 after the first three treatments. There was a statistically significant increase after each treatment, when compared to the proportion of PBMCs expressing CD25 before treatment. After 5 injections (the complete course of autohaemotherapy), there was a significant mean increase in the proportion of PBMCs expressing CD25, $P < 0.001$, n = 14. One week after the last treatment, the proportion of PBMCs expressing CD25 was still more than that before this treatment, $P < 0.05$, n = 6. Table 4-3 shows that no change occured in the control group. In Figure 4-2 a comparison between the treated and control groups shows a statistically significant increase in the proportion of PBMCs expressing CD25 after the second, third, fourth injection HOU-treated homologous blood and post-treatment mean ($P < 0.05$, < 0.02, < 0.05 and < 0.01 respectively).

Table 4-2. The expression of CD25 on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment Mean (n=5)	1 week post treatment
1	17	26	34	55	58	30	40.6	57
2	13	75	39	21	58	74	53.4	
3	29	69	72	27	100	45	62.6	
4	40	42	60	72	56	88	63.6	59
5	50	96	89	87	85	71	85.6	72
6	34	32	58	40	31	40	40.2	35
7	48	45	46	76	45	52	52.8	
8	50	53	77	63	56	82	66..2	
9	51	50	50	64	47	58	53.8	
10	47	51	41	46	36	38	42.4	
11	30	79	62	80	52	36	61.8	61
12	47	48	48	53	57	71	55.4	53
13	31	43	45	45	55	48	47.2	
14	40	42	46	60	43	45	47.2	
Mean	37.64	53.64	54.79	56.36	55.64	55.57	55.20	56.17
Sd	12.41	19.36	15.79	19.36	17.97	18.51	12.26	12.17
N	14	14	14	14	14	14	14	6
P*	—	< 0.05	< 0.002	< 0.01	< 0.02	< 0.01	< 0.001	< 0.05

P* values are calculated in comparison to the pre-treatment baseline.

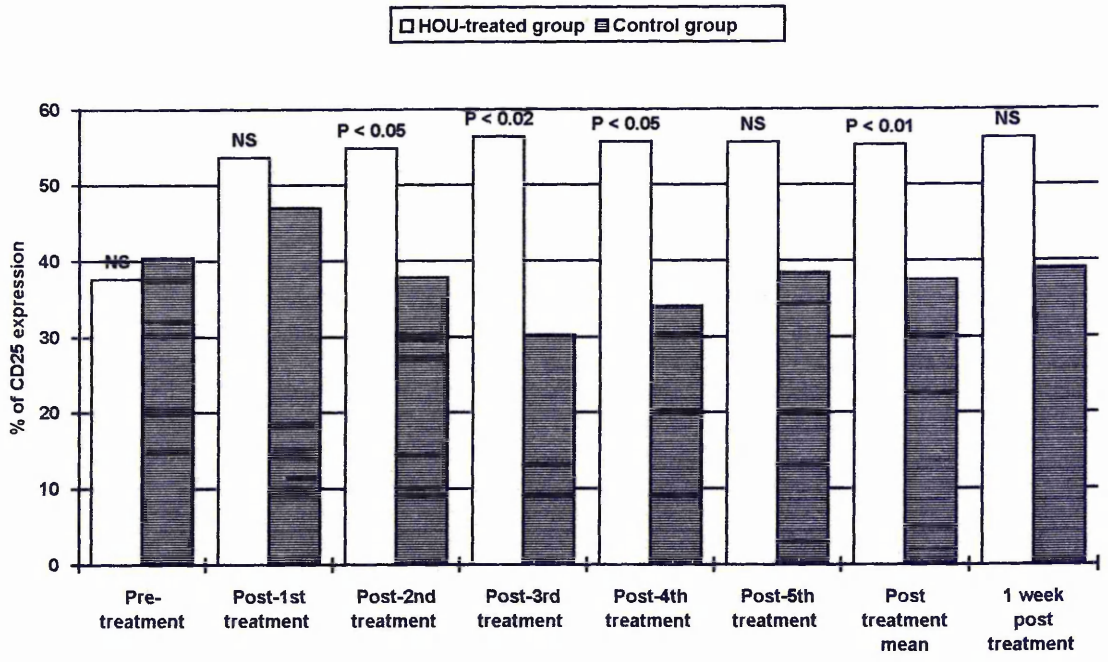
Table 4-3. The expression of CD25 on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in a control group of normal volunteers reinjected with their own untreated blood.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post reatment mean (n=5)	1 week post treatment
1	22	73	31	17	45	57	44.6	
2	25	39	35	30	21	34	31.8	
3	60	49	46	44	40	34	42.6	43
4	47	42	25	32	46	35	36.0	45
5	48	32	52	28	18	32	32.4	29
Mean	40.40	47.00	37.80	30.20	34.00	38.40	37.48	39.00
Sd	16.29	15.76	11.03	9.65	13.47	10.45	5.86	8.72
N	5	5	5	5	5	5	5	3
P*	—	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

Figure 4-2. Comparisons of CD25 expression on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in the HOU-treated and the control group of normal volunteers.



P values are calculated in comparison to the control group.

NS No statistically significant changes at $P > 0.05$.

4.3.1.2 Expression of HLA-DR on PBMCs

Table 4-4 shows the expression of HLA-DR assessed by the APAAP method on the PBMCs of healthy volunteers after autohaemotherapy with HOU-treated homologous blood. There was a gradual increase in the proportion of PBMCs expressing HLA-DR after the first three treatments. There was also a statistically significant increase after every treatment, when compared to the proportion of PBMCs expressing HLA-DR before treatment. After completing 5 treatments, there was a significant mean increase in the proportion of PBMCs expressing HLA-DR, $P < 0.001$, n = 14. One week after the last treatment, the proportion of PBMCs expressing HLA-DR was not significantly different from before this treatment ($P > 0.05$, n= 6). Table 4-5 shows that there were no significant changes in the control group. A comparison of the treated and control group in Figure 4-3 shows that there was a significant increases in the proportion of PBMCs expressing HLA-DR after every treatment and post treatment mean, $P < 0.05$, < 0.01, < 0.05, < 0.05, 0.02 and < 0.002 respectively.

Table 4-4. The expression of HLA-DR on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	34	34	45	54	50	49	46.4	39
2	16	37	33	35	55	82	48.4	
3	49	61	52	52	96	49	62.0	
4	25	42	35	75	54	91	59.4	60
5	26	57	76	92	39	62	65.2	79
6	48	44	71	35	53	75	55.6	46
7	41	44	45	70	55	65	55.8	
8	28	21	52	37	28	60	39.6	
9	46	53	73	56	54	62	59.6	
10	39	37	48	36	40	47	41.6	
11	40	67	52	70	40	47	55.2	68
12	45	48	52	59	61	60	56.0	44
13	39	37	48	36	40	47	41.6	
14	41	50	63	63	54	54	56.8	
Mean	36.93	45.14	53.21	54.86	51.36	60.71	52.70	56.00
Sd	9.83	12.03	13.18	17.82	15.75	13.81	8.50	15.63
N	14	14	14	14	14	14	14	6
P*	-----	< 0.05	< 0.001	< 0.01	< 0.01	< 0.001	< 0.001	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

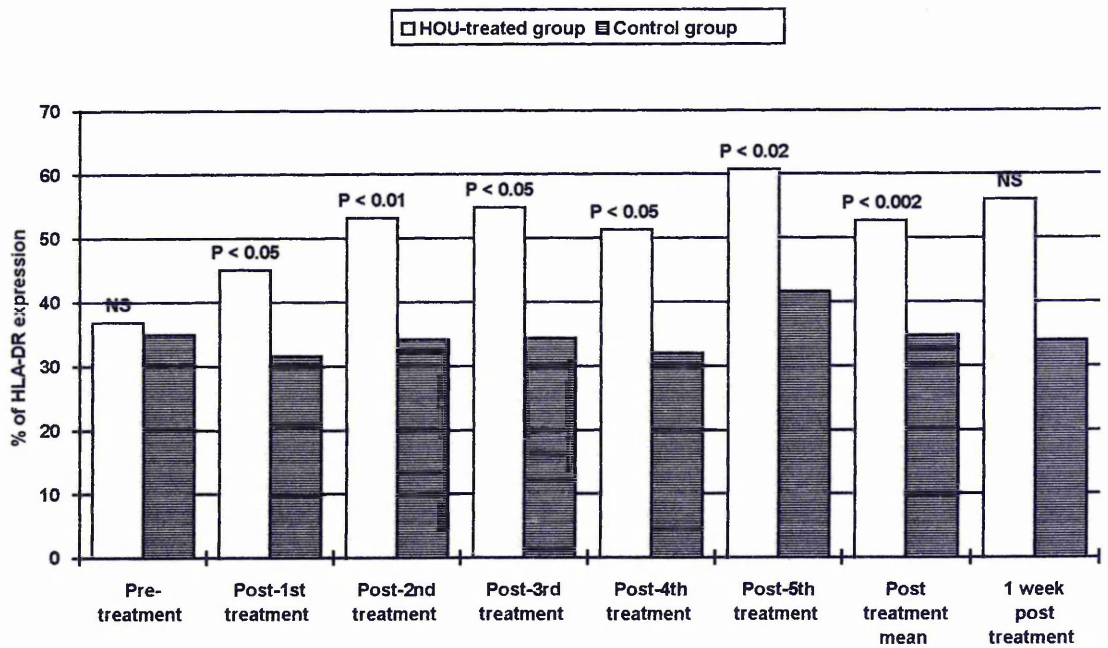
Table 4-5. The expression of HLA-DR on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in a control group of normal volunteers reinjected with their own untreated blood.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	33	30	37	39	47	56	41.8	
2	28	36	30	33	33	47	35.8	
3	55	33	43	51	35	40	40.4	31
4	20	15	19	17	14	24	17.8	29
5	39	44	42	32	31	41	38.0	42
Mean	35.00	31.60	34.20	34.40	32.00	41.60	34.76	34.0
Sd	13.17	10.64	9.93	12.32	11.83	11.72	9.75	7.0
N	5	5	5	5	5	5	5	3
P*	—	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

Figure 4-3. Comparisons of HLA-DR expression on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in the HOU-treated and the control group of normal volunteers.



P values are calculated in comparison to the control group.

NS No statistically significant changes at $P > 0.05$.

4.3.1.3 Expression of Ber-Mac3 on PBMCs

Table 4-6 shows the expression of Ber-Mac3 measured by the APAAP method on the PBMCs of healthy volunteers following a course of autohaemotherapy with HOU-treated homologous blood. There was a statistically significant increases after the first and second treatments ($P < 0.05$ and < 0.01 respectly), when compared to the proportion of PBMCs expressing Ber-Mac3 before treatment. After completing 5 treatments, there was a significant mean increase in the proportion of PBMCs expressing Ber-Mac3, $P < 0.01$, n = 14. One week after the last treatment, the proportion of PBMCs expressing Ber-Mac3 was not significantly different from the proportion of PBMCs expressing Ber-Mac3 before treatment ($P > 0.05$, n = 6). Table 4-7 shows that there were no statistically significant changes in the control group. In Figure 4-4 a comparison of the treated and control groups showed a significant difference in the proportion of PBMCs expressing Ber-Mac3 after the second treatment ($P < 0.05$, n=14).

Table 4-6. The expression of Ber-Mac 3 on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	41	37	52	25	34	47	39.0	26
2	19	31	37	32	34	18	30.4	
3	39	39	44	46	61	47	47.4	
4	39	42	55	60	33	65	51.0	29
5	19	40	47	34	37	16	34.8	32
6	46	49	52	35	38	25	39.8	29
7	30	30	35	34	37	46	36.6	
8	38	36	45	34	45	44	40.8	
9	38	42	37	40	32	40	38.2	
10	30	28	47	38	27	34	34.8	
11	34	58	35	44	44	40	44.2	40
12	37	48	47	31	56	70	50.4	44
13	29	38	58	69	32	47	48.8	
14	33	29	28	35	35	26	30.6	
Mean	33.71	39.07	44.21	39.86	38.93	40.36	40.49	33.33
Sd	7.80	8.51	8.72	11.81	9.56	15.79	6.94	7.09
N	14	14	14	14	14	14	14	6
P*		< 0.05	< 0.002	NS	NS	NS	< 0.01	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$

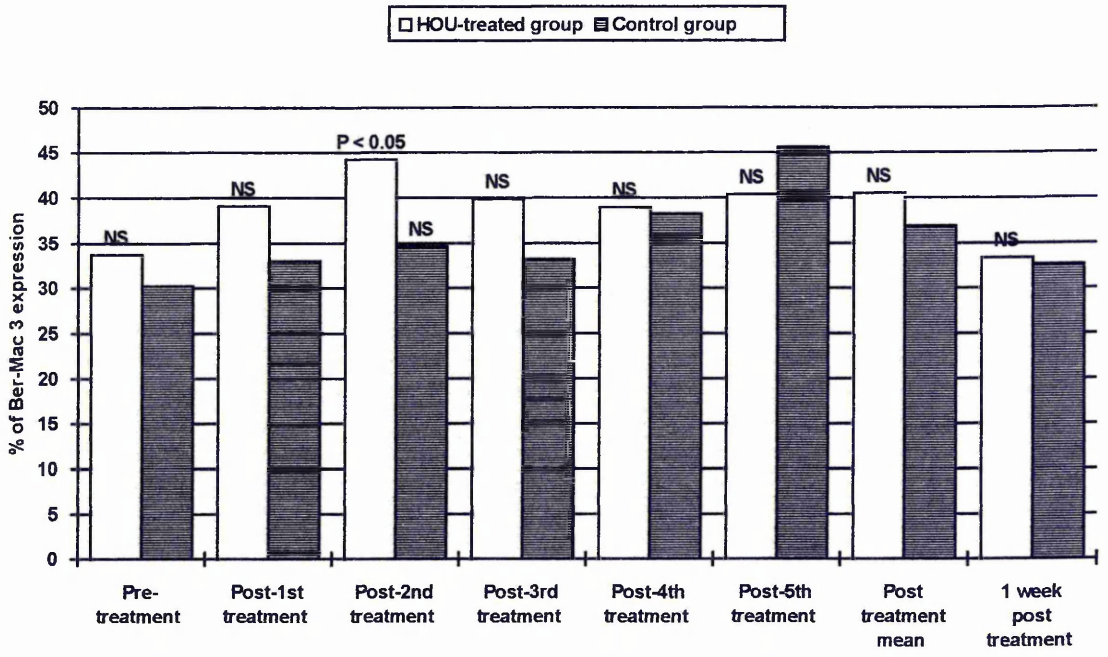
Table 4-7. The expression of Ber-Mac 3 on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in a control group of normal volunteers reinjected with their own untreated blood.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	29	21	25	27	26	31	26.0	
2	30	27	36	41	49	43	39.2	
3	35	76	38	33	54	68	53.8	48
4	19	21	36	29	39	64	37.8	28
5	38	20	38	36	23	22	27.8	22
Mean	30.20	33.00	34.60	33.20	38.20	45.60	36.92	32.67
Sd	7.26	24.20	5.46	5.59	13.66	20.11	11.11	13.61
N	5	5	5	5	5	5	5	3
P*	---	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

Figure 4-4. Comparisons of Ber-Mac 3 expression on peripheral blood mononuclear cells (as a percentage of CD45 positive cells) in the HOU-treated and the control group of normal volunteers.



P values are calculated in comparison to the control group.

NS No statistically significant changes at $P > 0.05$.

4.3.2 6-keto-prostaglandin F_{1a}

Table 4-8 shows the 6-keto-prostaglandin $F_{1\alpha}$ levels in plasma before, during and after a course of HOU-treatment. There were significant increase in 6-keto-prostaglandin F_{1a} concentrations after the first, the second and the fifth HOU-treatment compared with pre-treatment levels ($P < 0.05$, n = 14). After completling 5 treatments, there was a statistically significant mean increase in 6-keto-prostaglandin F_{1a} concentrations, $P < 0.02$, n = 14. One week after the last treatment, 6-keto-prostaglandin F_{1a} levels were not significantly increased compared to those measured before treatment ($P > 0.05$, n = 6). One year after the last treatment, the 6-keto-prostaglandin F_{1a} levels in the HOU-treated group showed a level not different from pre-treatment level (n = 13, pre-treatment: 27.6 pg / mL, 1 year later: 27.8 pg / mL). Table 4-9 shows that no change occured in the control group. In Figure 4-5 a comparison between the treated and control groups showed no significant differences in 6-keto-prostaglandin F_{1a} concentrations. Although one week after the last treatment, the mean of 6-keto-prostaglandin F_{1a} levels in HOU-treated group were much higher than that in control group, this did not reach a statistical significance probably as a result of too small a number of observations.

Table 4-8. 6-keto-prostaglandin $F_{1\alpha}$ concentrations (pg / mL) in the plasma from normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatmen t	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment	1 year post treatment
1	55.25	68.35	58.20	59.80	101.80	51.35	67.90	31.18	27.00
2	16.28	12.93	14.24	17.16	24.00	25.01	18.67		31.00
3	7.21	17.94	18.35	42.31	20.23	24.26	24.62		28.00
4	16.75	34.00	17.25	51.00	49.00	60.00	42.25	59.00	10.50
5	17.15	44.43	30.80	25.79	4.17	37.00	28.44	21.98	42.50
6	53.75	56.60	72.00	44.65	88.50	68.75	66.10	63.15	21.50
7	23.42	34.69	31.79	15.33	24.51	54.60	32.18		23.00
8	30.69	27.70	23.55	38.40	34.64	26.71	30.20		22.00
9	44.12	33.01	38.62	29.69	12.33	21.90	27.11		22.00
10	16.06	25.52	20.03	29.42	22.07	15.11	22.43		19.00
11	10.25	13.22	17.42	13.12	37.50	11.23	18.50	79.05	35.00
12	42.97	82.75	42.72	107.00	68.22	56.24	71.39	131.40	47.00
13	24.63	24.08	33.04	15.92	10.58	23.50	21.42		33.00
14	14.76	17.69	18.35	27.20	47.58	36.69	29.50		
Mean	26.66	35.21	31.17	36.91	38.94	36.60	35.76	64.29	27.81
Sd	16.06	21.06	17.03	24.67	29.42	18.37	18.76	39.08	9.92
N	14	14	14	14	14	14	14	6	13
P*	—	< 0.05	< 0.05	NS	NS	< 0.05	< 0.02	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

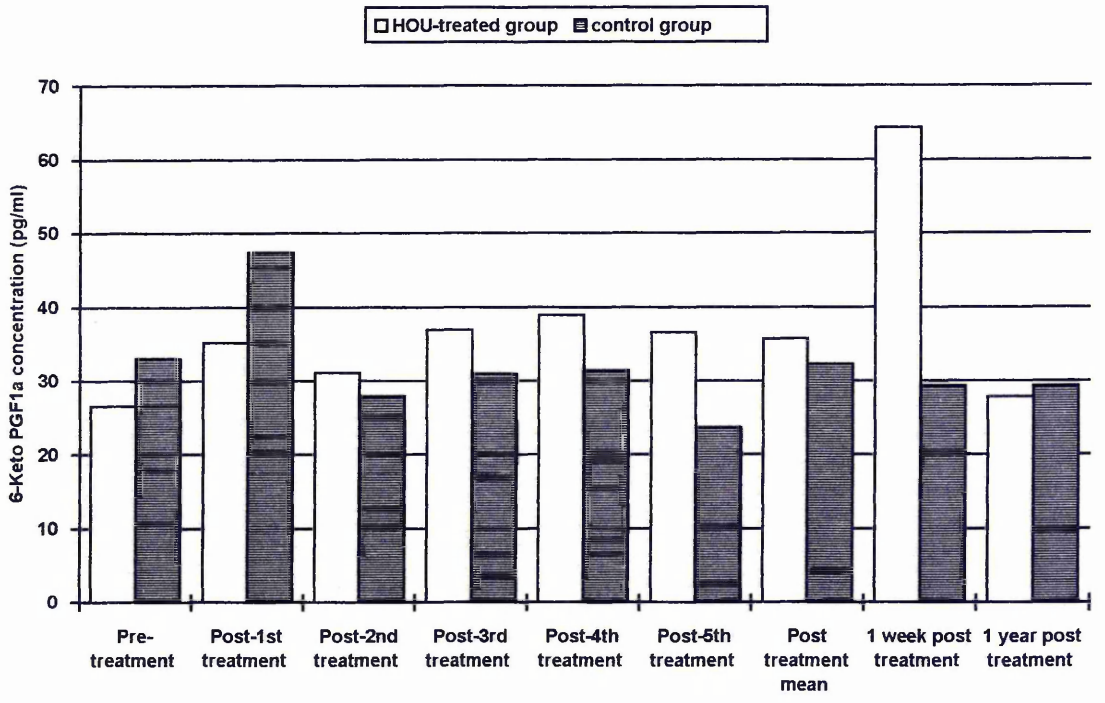
Table 4-9. 6-keto-prostaglandin $F_{1\alpha}$ concentration (pg / mL) in the plasma from control group of normal volunteers reinjected with their own untreated blood.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment	1 year post treatment
1	25.00	96.00	22.00	37.00	30.30	15.00	40.06		27.00
2	28.00	17.00	40.00	35.60	36.50	27.00	31.22		14.50
3	30.70	35.00	10.50	34.00	31.00	29.00	27.90	27.50	65.00
4	52.50	62.50	25.25	18.00	49.00	37.00	38.35	29.50	18.50
5	29.00	26.50	42.00	30.00	10.30	10.30	23.82	31.00	21.50
Mean	33.04	47.40	27.95	30.92	31.42	23.66	32.27	29.33	29.30
Sd	11.07	32.03	13.13	7.68	13.99	10.85	6.88	1.76	20.47
N	5	5	5	5	5	5	5	3	5
P*	—	NS	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

Figure 4-5. Comparisons of the concentration of 6-keto $PGF_{1\alpha}$ in the plasma from HOU-treated and the control groups of normal volunteers.



There were no statistically significant differences at $P > 0.05$.

4.3.3 IFN-gamma and IL-2

Table 4-10 shows the plasma levels of IFNγ in 10 healthy volunteers after autohaemotherapy with HOU-treated homologous blood. IFN-γ was not detectable in most of the samples.

Table 4-11 shows the plasma levels of IFNγ in 3 healthy volunteers in the control group. None of the samples had detectable levels of IFN-gamma.

The plasma levels of IL-2 was measured in 10 healthy volunteers after autohaemotherapy with HOU-treated homologous blood and 3 healthy volunteers in the control group. IL-2 was not detectable in most of the samples (not shown).

Table 4-10. IFN-gamma concentration (pg / mL) in the serum from the normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	1 week post treatment
2	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	
3	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	
5	5.55	< 5.0	8.35	5.3	< 5.0	< 5.0	< 5.0
7	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	
8	< 5.0	< 5.0	< 5.0	< 5.0	5.6	< 5.0	
9	< 5.0	< 5.0	< 5.0	< 5.0	5.2	< 5.0	
10	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	
11	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
13	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
14	6.0	< 5.0	< 5.0	7.6	< 5.0	< 5.0	
N	10	10	10	10	10	10	3

Table 4-11. IFN-gamma concentration (pg / mL) in serum from the control group of normal volunteers re-injected with their own untreated blood.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment
1	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
2	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0
5	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0	< 5.0

4.3.4 Nitrite plus nitrate

Table 4-12 shows the nitrite plus nitrate concentrations in the plasma from healthy volunteers following a course of autohaemotherapy with HOU-treated homologous blood. There were no significant differences in the nitrite plus nitrate concentrations after treatment, when compared to the nitrite plus nitrate concentrations before treatment. Table 4-13 shows the nitrite plus nitrate concentrations in the plasma from healthy volunteers of the control group. There were also no significant differences in the nitrite plus nitrate concentrations. A comparison bewteen the HOU-treated and control groups showed no significant difference in the nitrite plus nitrate concentrations (Figure 4-6).

Table 4-12. Nitrite plus nitrate concentration (as μM nitrite / mL) in the plasma from normal healthy volunteers treated with antologous blood exposed to HOU stimuli.

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	47.0	45.0	38.0	61.0	66.0	48.0	51.60	66.0
2	34.5	23.0	14.75	33.0	36.0	34.5	28.25	
3	72.0	40.0	56.0	56.0	55.0	37.0	48.80	
4	62.5	54.0	54.0	84.0	70.0	61.0	64.60	66.0
5	74.0	84.0	84.0	73.0	96.0	80.0	83.40	52.0
6	47.0	42.0	33.0	56.0	42.0	35.0	41.60	33.0
7	58.0	64.0	43.0	35.0	37.0	22.0	40.20	
8	39.0	35.0	31.0	23.0	34..0	25.0	29.60	
9	70.0	32.0	16.0	29.0	34.0	41.0	30.40	
10	62.0	27.0	45.0	58.0	45.0	60.0	47.00	
11	78.0	96.0	100.0	82.0	92.0	78.0	89.60	72.0
12	66.0	70.0	45.0	23.0	48.0	47.0	46.60	60.0
13	36.0	41.0	22.0	37.0	25.0	56.0	36.20	
14	34.0	34.0	66.0	58.0	60.0	60.0	55.60	
Mean	55.71	49.07	46.27	50.57	52.86	48.89	49.53	58.17
Sd	15.78	21.85	24.56	20.77	21.74	17.89	18.77	14.06
N	14	14	14	14	14	14	14	6
P*	----	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

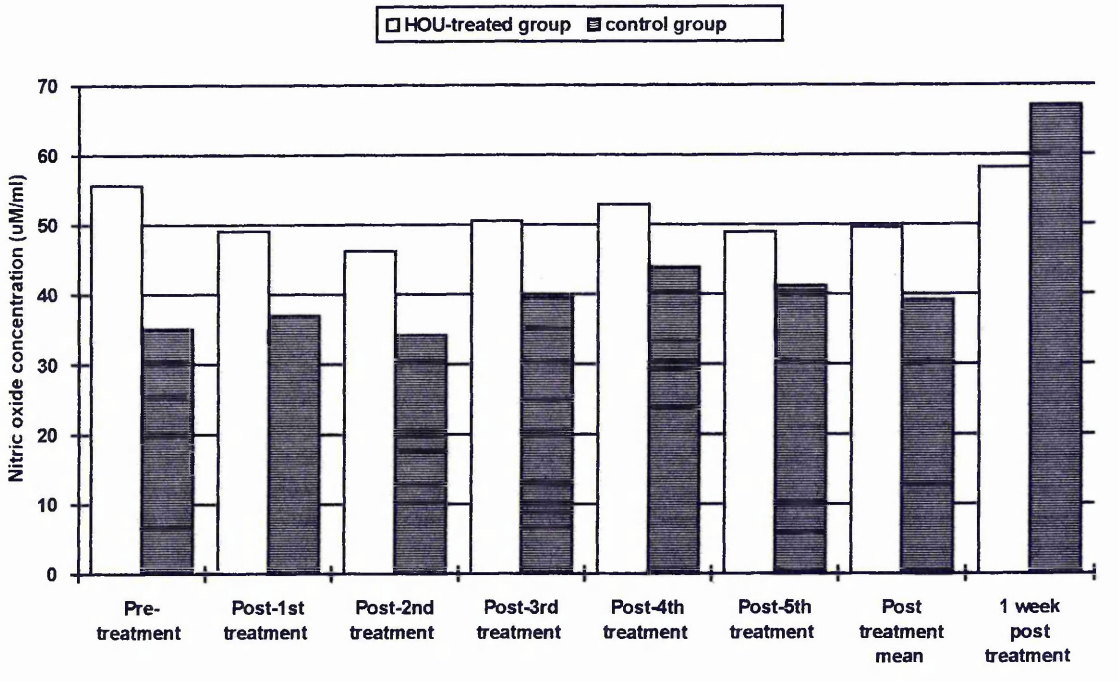
Table 4-13. Nitrite plus nitrate concentration (as μM nitrite / mL) in the plasma from a control group of normal healthy volunteers reinjected with their own untreated blood

Subject No	Pre-treatment (Baseline)	Post-1st treatment	Post-2nd treatment	Post-3rd treatment	Post-4th treatment	Post-5th treatment	Post treatment mean (n=5)	1 week post treatment
1	35.0	33.0	21.0	34.0	43.0	18.0	29.80	
2	26.0	18.0	30.0	42.0	39.0	31.0	32.00	
3	40.0	61.0	38.0	38.0	46.0	66.0	49.80	110.0
4	40.0	46.0	56.0	54.0	62.5	68.0	57.30	60.0
5	34.5	27.0	26.0	30.0	29.0	23.0	27.00	31.0
Mean	35.10	37.00	34.20	39.60	43.90	41.20	39.18	67.00
Sd	5.73	16.84	13.68	9.21	12.22	24.01	13.50	39.96
N	5	5	5	5	5	5	5	3
P*	—	NS	NS	NS	NS	NS	NS	NS

P* values are calculated in comparison to the pre-treatment baseline.

NS: No significant difference at $P > 0.05$.

Figure 4-6. Comparisons of the concentration of nitric oxide in the plasma from HOU-treated and control groups of normal volunteers.



There were no statistically significant differences at $P > 0.05$.

4.4 Discussion

The gene of the human major histocompatibility complex (MHC) Class II consists of some subregions: HLA-DP, -DQ and -DR, containing a minimum of one alpha and one beta chain. All HLA Class II gene subregion products show a similar biochemical structure. DAKO-HLA -DR, CR3 / 43 is a mouse monoclonal antibody that reacts with the beta-chain of all products of the gene subregions DP, DQ and DR. In peripheral blood DAKO-HLA -DR, CR3 / 43 stains activated mononuclear cells. Their expression by and the accompanying activation of macrophages is highly dependant on the release of IFNγ from activated T cells (Unanue, 1992; Basham & Merigan, 1983; Vivelizier, et al, 1984; McNicholas, et al, 1982). MHC complex Class II antigen expression is also commonly induced by TNFα, GM-CSF, IL-4, bacterial lipopolysaccharide (LPS) (Bendtzen et al 1988; Schwamberger et al, 1989; Phillips et al, 1990; Watanabe & Jacob, 1991), reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (Hibbs, et al, 1988; Liew & Cox, 1991). The results presented above show that autohaemotherapy with HOU-treated blood enhances the expression of HLA-DR on lymphocytes and monocytes as measured by APAAP method. This may be due to the fact that after HOU-autohaemotherapy, upon blood reinjection, PBMCs activated *ex vivo* by HOU can stimulate other PBMCs to release cytokines including IFNγ, TNFα, GM-CSF and reactive nitrogen intermediates (RNI).

IL-2R is a multi-subunit system consisting of a 55 kD α-subunit (also known in the human as Tac antigen) and 70 kD β-subunit. Whereas the β-subunit is expressed on resting T cells, the α-subunit expression must be induced by mitogenic stimulation or IL-2 stimulation, i.e. the α-subunit is strongly expressed on activated T cells (Greene, et al, 1986; Morgan et al, 1976). The mouse mAb DAKO-CD25, ACT-1 reacts with the α-subunit of IL-2R. In this *in vivo* trial, the result shows that after autohaemotherapy with

HOU-treated blood there was an increase in the expression of IL-2R on T lymphocytes as measured by the APAAP method. This suggests that upon HOU-treated blood reinjection, *ex vivo* activated T cells may activate the other peripheral blood T cells via IL-2 release and these activated T cells may release more IL-2 to stimulate more T cells to activate.

Ber-Mac3. is a 140 KD glycoprotein and expressed in high density on lipopolysaccharide or IFN-γ stimulated monocytes (Backe et al, 1991). The results presented in this investigation show that autohaemotherapy with blood exposured to HOU enhances the expression of Ber-Mac3 on monocytes. This implies that after HOU-autohaemotherapy, T cells can be activated and may release IFN-γ to activate peripheral blood monocytes.

The above observation of the expression of some activation markers on PBMCs suggest that autohemotherapy with HOU-treated blood may modify cytokine levels.

Recently, a study of leukocyte-endothelial cell interactions suggested that many agonists, such as the cytokines IFNγ, TNFα, IL-1 and IL-2 secreted from activated leukocytes, as well as lipopolysaccharide (LPS), can activate the endothelial cells and stimulate nitric oxide synthesis (Li, et al, 1991; Zimmerman, et al, 1992). NO is synthesised from L-arginine by NO synthase (NOS) and in the endothelial cells, there are two types of NOS, calcium dependent (caNOS) and calcium independent (ciNOS) isoforms, the former is constitutive and the latter is an inducible isoform of NOS (Moncada, et al, 1991).

However, in this *in vivo* study the levels of IL-2 and IFN-γ in the peripheral blood circulation were mostly undetectable and there was no significant increase in the levels of nitric oxide after autohaemotherapy with blood treated with HOU. This could be because, upon reinjection, the fractions of PBMCs activated by heat, ozone and

ultraviolet, home in on lymphoid microenvironments, such as spleen, bone marrow and lymph nodes and non-lymphoid microenvironments and there begin to release cytokines and nitric oxide that interact with either stationary cells or by PBMCs in transit. The current evidence is tending toward the view that in physiological conditions cytokines and nitric oxide are usually made as autocrine and paracrine factors secreted into a limited space between interacting cells, and any small amounts reaching the circulation do not disturb homoeostasis (Jacob, 1989; Bocci, 1988; Liew & Cox, 1991). For each reinjection of PBMC in the small 10 ml HOU-treated blood this only represents a minimal fraction of the whole PBMC population of the internal milieu. Therefore, unless a far larger number of cells is involved, the spillovers of cytokine and NO in the circulation appear unlikely but, even if it occurs to a small extent, dilution in the plasma pool and rapid turnover would prevent a significant increase (Bocci, 1988).

Furthermore, levels of nitrate and nitrite in blood can be affected by a number of factors including dietary intake and renal clearance. Thus, these measurements are at best an imprecise reflection of nitric oxide production.

The above *in vivo* results in which little or no IFNγ and IL-2 could be detected in the peripheral circulation of treated or control volunteers are compatible with those of Bocci, et al. Bocci and his colleagues (1994) demonstrated that after a single or five consecutive autohaemotherapy treatments with ozonized blood, there was a lack of circulating IL-1β, IL-2, IL-6, TNFα, IFNβ, IFNγ and GM-CSF, but there was an increase in the levels of Mx protein, a marker of cell activation, in blood. The expression of Mx protein in PBMC is induced by IFNα and is a fairly long-lasting process in comparison to the turnover of cytokines (Bocci, et al, 1994).

The above results show that in a group of young health volunteers, treatment by

reinjection of HOU-treated blood produces a significant rise in prostacyclin as measured as its stable metabolite 6-keto-$PGF_{1\alpha}$. The primary site of prostacyclin synthesis is the endothelium of blood vessels (Vane & Botting, 1995). The generation of prostacyclin by endothelial cells can be stimulated by contact with activated leukocytes (Vane, et al; 1990). Hakkert and his colleagues (1992) have reported that direct cell-cell contact between purified monocytes and endothelial cells strongly enhanced PGI_2 synthesis by endothelial cells. Secretory products, such as IL-1, from monocytes and neutrophils, also increase the level and the expression of cyclooxygenase mRNA in endothelial cells and enhance endothelial PGI_2 synthesis, although to a lesser extent than with monocytes that were in direct contact with endothelial cells. Monocytes produce and secrete arachidonic acid metabolites by the cyclooxygenase pathway (Hakkert, et al, 1992). Thromboxanes are the major products synthesized by monocytes via this route, whereas PGI_2 is produced only in small amounts. It is possible that monocytes in close contact with endothelial cells enhance the endothelial PGI_2 production by providing the endothelial cells with exogenous eicosanoids (Hakkert, et al, 1992).

In conclusion it appears that autohaemotherapy with HOU-treated blood in normal subjects leads to a very mild process of activation of the immune system, without apparent side effects because of a lack of leakage of cytokines into the general circulation. Possibly by the interaction between endothelial cells and activated leukocytes or by cytokines secreted by activated leukocytes, the endothelium of blood vessels becomes activated and then increases in the synthesis of prostacyclin or nitric oxide. Prostacyclin and nitric oxide are known to dilate blood vessels (Moncada & Vane, 1979; Palmer, et al, 1988; Stuehr, et al, 1989) and inhibit platelet aggregation (Radomski et al, 1987 [1] [2] [3]). Such increases of prostacyclin and nitric oxide would therefore offer a dual beneficial effect in the treatment of vascular disease.

The results from this *in vivo* study provide the first objective evidence and support the subjective anecdotal observation of clinical benefits in peripheral vascular disease following ozone/oxygen autohaemotherapy or HOU autohaemotherapy.

CHAPTER 5

Summary and Conclusions

Atherosclerosis and its complications, such as myocardial infarction, stroke and peripheral vascular disease, remain major causes of morbidity and mortality in the Western World (Munro & Cotran, 1988; Fowkes, 1988; Office of Population Censuses & Surveys, 1991). At the present time there is no widely accepted successful drug treatment (Lowe, 1990; Lutomski, et al, 1995) for atherosclerosis. Atheroslerosis is the commonest and most important vascular disease (Fowkes, 1990; Leng, 1995). With the recent advance in cellular immunology, atherosclerosis has been recognised as an immunologically mediated disease (Wick, et al, 1995). The lesions of atherosclerosis result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium of the artery wall. A large number of growth factors, cytokines and vasoregulatory molecules participate in this process (Ross, 1993).

Although ozone is toxic to pulmonary tissue when inhaled as an environmental pollutant (Hatch, et al, 1990), the insufflation of blood with oxygen containing low levels of ozone gas alone, or in the presence of heat and UV, and re-injection into the donor (ozone/oxygen autohaemotherapy or HOU-autohaemotherapy) has been used for a long time in continental Europe for the treatment of a variety of diseases, and in particular of peripheral vascular disease (Rilling & Viebahn, 1987; Gierek, et al, 1992; Romero, et al, 1993 [1] [2]). In Germany, for example, approximately 380,000 patients have been treated.

The claims for the beneficial effects have already been outlined in Chapter 1. Currently these studies have provided an explanation of the some mechanisms of the action of HOU-autohaemotherapy.

This study has investigated the effect of the treatment of blood *ex vivo* with HOU on the platelets *in vitro*. The results show that either in the healthy volunteers or in patients with PVD and / or diabetes, there is a significant inhibition of platelet aggregation in response to various agonists (ADP, thrombin and the calcium ionophore A23187) when whole blood is treated with HOU. That inhibition of platelet aggregation does not result from the disruption of the integrity of the platelets is indicated by the evidence that platelets isolated from treated blood aggregated normally in platelet poor plasma isolated from untreated blood. Furthermore, there were no significant differences in the levels of PDGF, present in platelet α-granules, observed between the HOU-treated blood and the untreated blood.

NF (nuclear factor)-kB is thought to be an oxidative stress-responsive transcription factor of eukaryotic cells and can activate a great variety of genes involved in defence reactions (Schreck et al, 1992). NF-kB activation can also be induced by UV irradiation. *In vitro* HOU treatment of blood may activate NF-kB in the leucocyte populations, resulting in the upregulation of cytokine synthesis. Many *in vitro* experiments have proved that ozone induces peripheral blood mononuclear cells (PBMCs) to release cytokines, including IFNβ, IFNγ, TNFα, IL-1β, IL-2, IL-6 and GM-CSF (Bocci & Paulesu, 1990; Paulesu, et al, 1991; Bocci, et al, 1994).

It has been reported that IFNγ, TNFα, IL-1 and IL-2, can stimulate monocytes and neutrophils to produce NO by upregulating nitric oxide synthase, which exists as an inducible isoform in these cells (Salvemini et al, 1989; Liew & Cox, 1991; Moncada & Palmer, 1991; Vallance & Collier, 1994; Ding, et al, 1988; Hibbs, et al, 1987; 1992; McCall, et al, 1990; Stuehr, et al, 1985; 1989; Wright, et al, 1989). In the *in vitro* HOU-treated whole blood there was a significant increase in the concentration of nitric oxide, as measured by its stable metabolites nitrite plus nitrate.

Many results indicate that nitric oxide plays a role in the inhibition of platelet aggregation and in vasodilation, by the stimulation of soluble guanylate cyclase and the induction of a rise in the intracellular levels of cyclic GMP in platelets and vascular smooth muscle cells (Ignarro, et al, 1987; Ignarro, 1990; Synder & Bredt, 1992; Moncada, et al, 1989; Marletta, et al, 1988). NO has also been observed to inhibit mitogenesis and the proliferation of cultured rat vascular smooth muscle cells (Garg & Hassid, 1989).

Evidence that the observed inhibition of platelet aggregation in HOU-treated blood is at least in part caused by an increase in NO levels is provided by the reversal of the inhibition in the presence of oxyhaemoglobin, an NO inhibitor (Synder & Bredt, 1992; Salvimini et al, 1990; Ignarro, 1989 [1]; Sarrel, et al, 1990).

Recent research has indicated that monocytes can produce PGI_2 in small amounts by the cyclooxygenase pathway and that IL-1, secreted from activated monocytes and neutrophils, can enhance monocyte PGI_2 synthesis (Hakkert, et al, 1992). In this *in vitro* study the results showed an increase in the concentrations of prostacyclin, as measured by its stable metabolites 6-keto $PGF_{1\alpha}$, in the HOU-treated blood. The explanation for the increase in prostacyclin may be that the UV, ozone/oxygen and IL-1 released by the activated PBMC and neutrophils by HOU induce the synthesis of cycloxygenase by activating NF-kB.

Prostacyclin has been shown to inhibit platelet aggregation and induce vasodilatation by stimulating adenylate cyclase and inducing an elevation in intracellular levels of cyclic AMP in vascular smooth muscles and platelets (Radomski et al (2) (3), 1987; Moncada & Vane, 1979; Vane & Botting, 1995).

It is possible that activated monocytes and T lymphocytes in blood treated with HOU *in vitro*, after reinjection in the donor, may activate other peripheral leukocytes via

autocrine and paracrine effects and release IFNγ and IL-2. This is supported by *in vivo* results of the enhancement of the expression of the activation markers IL-2R, HLA-DR and Ber-Mac3 on PBMCs after autohemotherapy with HOU-treated blood.

The primary sites of prostacyclin (PGI_2) synthesis are the endothelium of blood vessels (Ross, 1993; Vane & Botting, 1995). The generation of prostacyclin by endothelial cells can be stimulated by contact with activated leukocytes (Vane, et al; 1990). Hakkert et al have demonstrated that *in vitro* direct cell-cell contact between monocytes and endothelial cells strongly enhances PGI_2 synthesis by the endothelial cells via the monocytes providing the endothelial cells with exogenous eicosanoids. Secretory products, such as IL-1, from monocytes and neutrophils, also increase the expression of cyclooxygenase mRNA in endothelial cells and enhances endothelial PGI_2 synthesis (Hakkert, et al, 1992). In the *in vivo* HOU studies the results show that after HOU-autohaemotherapy there is a significant increase in prostacyclin measured as its stable metabolite 6-keto-$PGF_{1\alpha}$, perhaps reflecting such an interaction between activated monocytes and the endothelium.

It has been discovered that in the endothelial cells there are two types of nitric oxide synthase, calcium dependent (caNOS) and calcium independent (ciNOS) isoforms, the former is constitutive and the latter is an inducible isoform of NOS (Moncada, et al, 1991; Marsden, et al, 1993). Many agonists, such as IFNγ, TNFα, IL-1 and lipopolysaccharide (LPS), can activate the endothelial cells and stimulate nitric oxide synthesis (Li, et al, 1991; Zimmenman, et al, 1992). In this *in vivo* study the enhancement of the expression of IL-2R, HLA-DR and Ber-Mac3 on PBMC after autohaemotherapy with HOU-treated blood suggests an increase in activation of T lymphocytes, which may release IFNγ. This would potentially enhance nitric oxide synthesis via stimulating inducible isoform of NOS in endothelial cells.

Overall, it appears that in normal subjects autohaemotherapy with HOU-treated blood leads to a very mild activation of the immune system. By an interaction between endothelial cells and activated leukocytes, or by the effect of cytokines secreted by activated leukocytes on endothelial cells, there is an increase in the synthesis of prostacyclin and / or nitric oxide by the endothelium. Prostacyclin and nitric oxide are known to dilate blood vessels (Moncada & Vane, 1979) and inhibit platelet aggregation (Radomski et al, 1987 [1] [2] [3]). So the increases of prostacyclin and nitric oxide might offer a dual beneficial effect in the treatment of vascular disease.

It has been stated that endothelial dysfunction plays a critical role in peripheral vascular disease (Vane, et al, 1990; Rau, 1991; Ware & Heistad, 1993; Ross, 1993). There is much evidence that a deficiency in the release of nitric oxide and prostacyclin by endothelial cells contributes to vascular disease (Rees, et al, 1989 [1] [2]; Vallance, et al, 1989; Panza, et al, 1990; Tolins & Shultz, 1994). If autohaemotherapy with HOU-treated blood did result in the upregulation of endotheliual production of vasodilators such as PGI_2 and NO, the therapy could be a major advance in the treatment of a number of vascular disease.

There were no apparent adverse effects of this treatment observed in these healthy volunteers as monitored by clinical symptoms, signs, urinary test, standard haematology screen and clinical chemical profile.

These observations provide objective evidence and support the subjective anecdotal observations of the clinical benefits of HOU-autohaemotherapy and similar ozone / oxygen autohaemotherapy in the treatment of peripheral vascular disease.

CHAPTER 6

Future Research

With the recent increase in knowledge of the underlying mechanisms of vascular disease and immunology, it has been possible to further evaluate autohaemotherapy with HOU-treated blood within the molecular biological, immunological and clinical areas. It is apparent that such an approach is necessary, in order for this treatment to become accepted by those responsible for treating patients.

1. Extension of the studies *in vitro*

To optimise the conditions of HOU stimulation, it is necessary to investigate the effects of time, temperature and ozone concentration. There may be different effects on the inhibition of platelet aggregation and on molecular marker expression on PBMCs, which can be more accurately estimated by flow cytometry than by the visual microscopic method used here. It has been reported that ozone at high concentrations can be noxious to cell components, for example, increasing the ozone concentration may produce a consistent increase of hemolysis due to a decrease in reduced glutathione, which maintains RBC membranes in a stable state (Bocci, et al, 1993 [2]) and a reduced production of IFN and IL-2 in conjunction with reduced PBMC respiration (Bocci & Paulesu, 1990; Bocci, et al, 1993 [1]). It is necessary to standardise the ozonization procedure in order to yield the maximal therapeutic effects with minimal damage to the cells.

In order to understand the process more fully, the identification of these populations of blood cells which are stimulated by this technique need to be determined in greater detail.

2. Extension of the studies *in vivo*

The results of this research so far indicate that there is a potential rationale for the application of HOU-autohaemotherapy in the area of occlusive vascular diseases (from critical limb to heart, brain and retinal ischaemia). This conclusion is derived from the observation, both *in vitro* and *in vivo*, of the increased production of prostacyclin and nitric oxide which are known to be inhibitors of blood vessel constriction and blood platelet aggregation and, which therefore improve blood flow.

It would be necessary to carry out clinical trials in patients with vascular disease and this study so far has provided data on the safety of the procedure. In future clinical trials in patients the clinical improvement should be assessed by strict clinical criteria relating to objective measurements and not limited to subjective assessments. The results of a double-blind, randomised control trial are also required in order to exclude a spontaneous remission and / or a placebo effect.

CHAPTER 7

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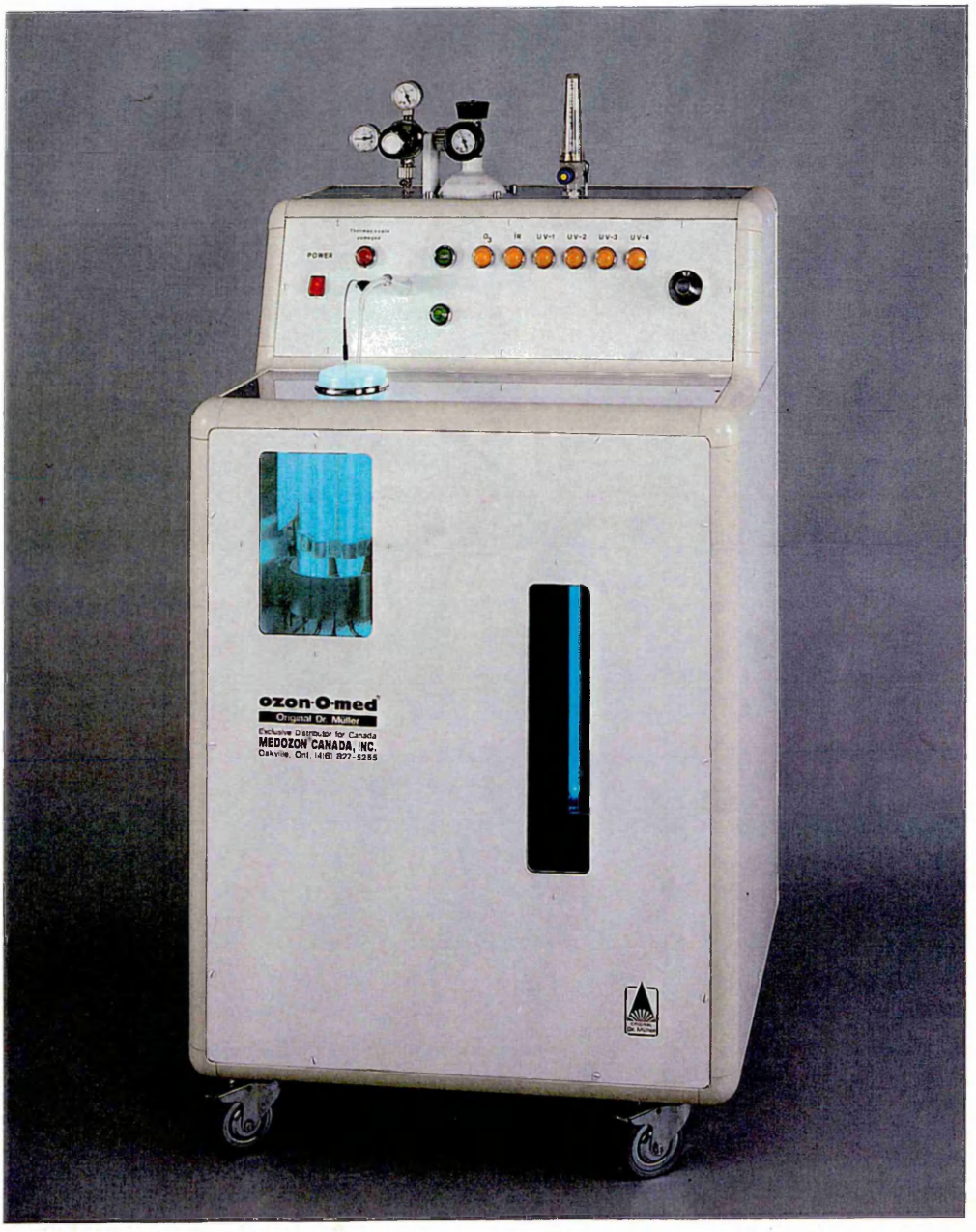
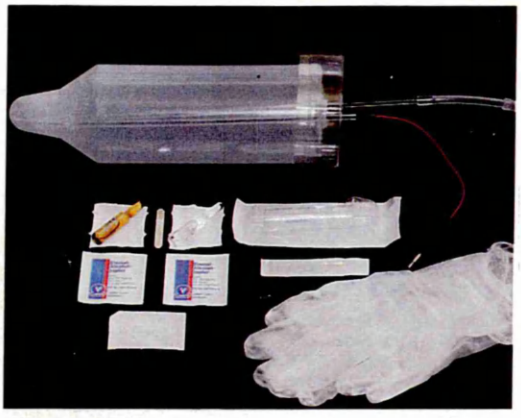
Appendices

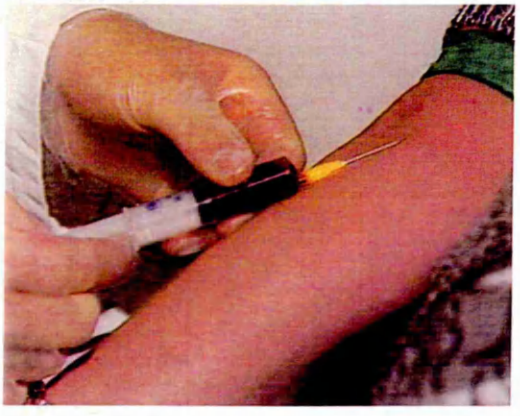
2-1. OZON-O-MED machine



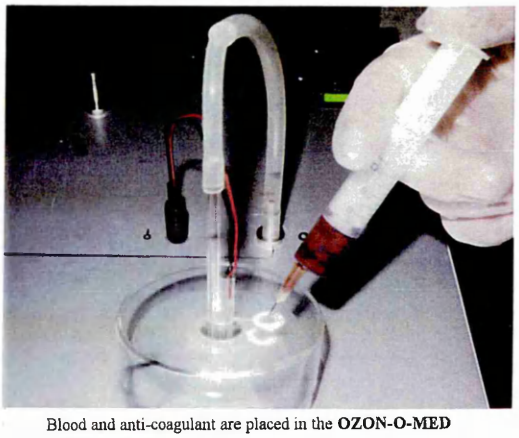
2-2. OZON-O-MED treatment process



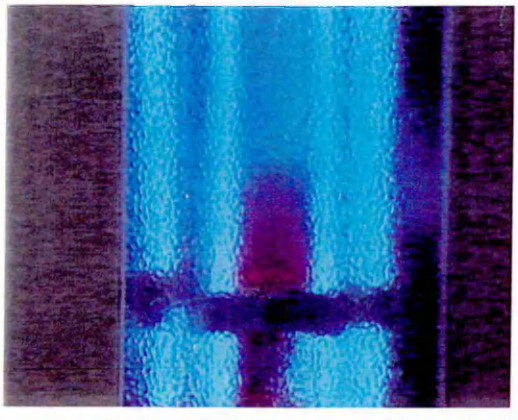
Disposable blood-handling kit (from BASF) for use with the **OZON-O-MED** machine.



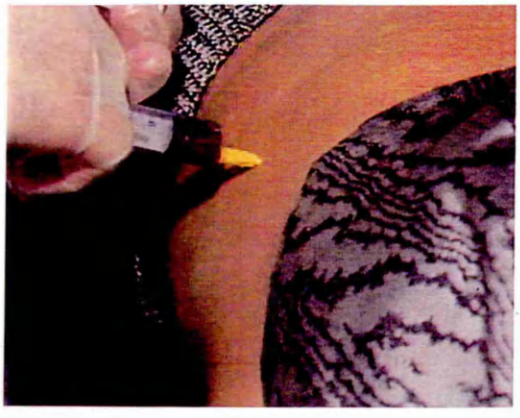
Patient's blood is drawn.



Blood and anti-coagulant are placed in the **OZON-O-MED** reaction tank.



Blood is ozonized, irradiated and heated inside the **OZON-O-MED** machine.



Re-injection of blood into patient.

2-3. Procedure for measurement of nitrite and nitrate

A reduction column was constructed from Teflon tubing threaded with a 1-meter length of 1-mm diameter cadmium wire. The column was prepared for use by washing with 10 ml quantities of 1 N HCl, distilled water, 2% copper sulphate solution, and distilled water in that sequence at about 20 ml per minute.

The Griess reagent consists of 1 part 0.1% naphthylethylene diamine dihydrochloride in distilled water plus 1 part 1% aminobenzenesulfonaminde in 5% concentrated H_3PO_4, the 2 parts being mixed together within 12 hours of use and kept chilled.

A 5% aqueous NH_4Cl adjusted to PH 9.0 with sodium borate was made and stored refrigerated for up to several months.

PPP was deproteinized before analysis as follows. To 250 μl of PPP was added 50 μl 35% sulfosalicyclic acid. Treated PPP was mixed by vortexing every 5 min and allowed to react for 30 min at room temperature. The treated samples were then centrifuged at 10,000 xg for 15 min.

A series of standard sodium nitrate solution was prepared. To two hundred microliters of the supernatant or standard nitrate solution was added 300 μl of 5% aqueous NH_4Cl buffer and 60 μl 5% NaOH and the mixture was passed through the cadmium column .

Nitrate in a sample is reduced by the cadmium column to $[NO_2]^-$ which reacts with the Griess reagent to form a purple azo dye. Nitrite in the sample needs no reduction. The colour of the product is developed at 60°C in a water bath, then cooled to 0°C, and its absorbance at 546 nm measured with a spectrophotometer (Green et al, 1982). The concentration of nitrite plus nitrate in PPP can be read off the standard curve of $[NO_3]^-$.

2-4. Extraction of 6-keto-prostaglandin F_{1a}

Solid phase extraction with Amprep C_2, 100 mg minicolumn (Amersham codes RPN 1903) was employed.

1) To condition the column

i) Rinse an Amprep C_2 100 mg minicolumn with 2 ml methanol.

ii) Rinse the column with 2 ml distilled water.

Note: Do not allow the sorbent in the column to dry.

2) Sample treatment

Acidify 1 ml of plasma with 100 μl of 2 M / L citric acid and apply to the column.

3) Remove interferences

i) Rinse the column with 5.0 ml distilled water.

ii) Rinse with 5.0 ml 10% ethanol.

iii) Rinse with 5.0 ml hexane or petroleum ether 30°C-40°C

4) Elution

Pass 5.0 ml methy formate through the column and collect the eluate.

The sample can be dried under nitrogen, reconstituted in assay buffer and assayed directly.

2-5. Radioimmunoassay for 6-keto prostaglandin-$F_{1\alpha}$

2-5-1. Principle of the radioimmunoassay for 6-keto prostaglandin-$F_{1\alpha}$

The concentration of 6-Keto prostaglandin-F1-alpha in the extraxted sample was measured using a commercially available radioimmunoassasy kit (Amersham International UK).

The assay is based on the competition between unlabelled 6-keto-prostaglandin F_{1a} and a fixed quantity of a high specific activity 6-keto-prostaglandin F_{1a} [I^{125}] iodotyrosine methyl ester tracer compound for binding to an antibody for 6-keto-prostaglandin F_{1a}. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound by the antibody will be inversely proportional to the concentration of added non-radioactive ligand.

Free		**Bound**
[^{125}I] 6-keto-prostaglandin F_{1a}		[^{125}I] 6-keto-prostaglandin F_{1a}-antibody
	+ antibody $\rightleftharpoons$	
6-keto-prostaglandin F_{1a}		6-keto-prostaglandin F_{1a}-antibody

The antibody bound 6-keto-prostaglandin F_{1a} is then reacted with Amerlex-M second antibody bound to magnetisable polymer particles. Separation of the antibody bound fraction is effected by centrifugation of the Amerlex-M suspension and decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled

6-keto-prostaglandin F_{1a} in the bound fraction to be calculated. The concentration of unlabelled 6-keto-prostaglandin F_{1a} in the sample is then determined by interpolation from a standard curve. This provides a rapid, simple and sensitive method for the determination of 6-keto-prostaglandin F_{1a} *in vitro* over the range 3-400 pg / tube.

2-5-2. Procedure of the radioimmunoassay for 6-keto-prostaglandin $F_{1\alpha}$

(1) Prepare all reagents and standards as the instructions.

(2) Equilibrate all reagents to room temperature, and mix before use.

(3) Label polypropylene tube in duplicate for total count tube (TC), non-specific binding tubes (NSB), zero standard tube (B_0), standards and samples.

(4) Pipette 300 μl of assay buffer into the zero standard tubes(B_0).

(5) Pipette 400 μl of assay buffer into the non-specific binding tubes(NSB).

(6) Pipette 200 μl of assay buffer into all standard and sample tubes.

(7) Pipette 100 μl of each diluted standard into the appropriatedly labelled tubes and vortex mix.

(8) Pipette 100 μl of each sample, in duplicate, into the appropriatedly labelled tubes and vortex mix.

(9) Pipette 100 μl of [^{125}I] 6-keto-prostaglandin F_{1a} into all tubes and vortex mix.

(11) Pipette 100 μl of antiserum into all tubes except the non-specific binding tubes(NSB).

(12) Vortex mix all tubes thoroughly for 2-5 seconds and incubate overnight (between 15 and 18 hours) at 2°C-8°C.

(13) Gently shake and swirl the bottle containing Amerlex-M second antibody reagent (blue-green) to ensure a homogenous suspension. Then add 500 μl to each tube except the TC.

(14) Vortex mix all tubes thoroughly and incubate for 10 minutes at room temperature (15°C-30°C).

(15) Seperate the antibody bound fraction by centrifugation for 10 minutes at 1500 g, then aspirate the supernatant liquids.

(16) Determine the radioactivity present in each tube by counting for at least 60 seconds in a gamma scintillation counter.

2-6. Height (mm) of washed platelet aggregation in HOU-treated whole blood in 5 individuals

(1) The first individual

Reagents	PPP[c] + PLT [c]	PPP[c] + PLT [t]	PPP[t] + PLT [t]	PPP[t] + PLT [c]
ADP (μM / L)				
100	66	130	110	50
50	30	50	6	6
25	25	58	30	25
10	19	13	15	10
Thrombin (u / ml)				
10	110	160	145	100
5	40	49	39	19
Calcium ionophore A23187 (μg / ml)				
100	28	30	25	24
50	19	20	5	5
5	20	21	19	10
Mean	39.7	59	43.8	27.7
Sd	30.3	51.7	49.5	30.5

(2) The second individual

Reagents	PPP[c] + PLT [c]	PPP[c] + PLT [t]	PPP[t] + PLT [t]	PPP[t] + PLT [c]
ADP (μM / L)				
100	65	45	33	5
50	120	52	52	33
25	20	30	45	25
10	15	40	38	10
Thrombin (u / ml)				
10	62	55	38	49
5	130	70	35	10
Calcium ionophore A23187 (μg / ml)				
100	10	33	15	40
50	10	10	7	10
5	8	10	0	10
Mean	48.9	38.3	29.2	21.3
Sd	48.4	20.1	17.7	16

(3) The third individual

Reagents	PPP[c] + PLT [c]	PPP[c] + PLT [t]	PPP[t] + PLT [t]	PPP[t] + PLT [c]
ADP (μM / L)				
100	55	20	4	4
50	25	45	12	15
25	15	42	5	5
10	20	15	5	8
Thrombin (u / ml)				
10	135	135	16	12
5	65	35	35	14
Calcium ionophore A23187 (μg / ml)				
100	43	25	25	27
50	10	30	26	9
5	27	20	18	12
Mean	43.9	40.8	16.2	11.8
Sd	38.8	36.8	10.9	6.9

(4) The fourth individual

Reagents	PPP[c] + PLT [c]	PPP[c] + PLT [t]	PPP[t] + PLT [t]	PPP[t] + PLT [c]
ADP (μM / L)				
100	45	13	5	10
50	20	15	5	5
25	8	18	4	8
10	14	14	5	5
Thrombin (u / ml)				
10	120	120	25	15
5	37	37	33	20
Calcium ionophore A23187 (μg / ml)				
100	25	4	20	14
50	12	16	30	20
5	30	9	5	15
Mean	34.6	27.3	14.7	12.4
Sd	34.2	35.9	12.2	5.8

(5) The fifth individual

Reagents	PPP[c] + PLT [c]	PPP[c] + PLT [t]	PPP[t] + PLT [t]	PPP[t] + PLT [c]
ADP (μM / L)				
100	70	42	88	57
50	55	42	44	49
25	67	42	29	42
10	42	33	10	45
Thrombin (u / ml)				
10	80	80	47	67
5	59	53	48	86
Calcium ionophore A23187 (μg / ml)				
100	38	8	10	22
50	10	0	20	26
5	22	19	12	14
Mean	49.2	35.4	34.2	45.3
Sd	23.1	24.3	25.6	22.9

Summary: The comparison of height of platelet aggregation (mm)

	A	B	C	D
Subject	**PLT[c] in PPP [c]**	**PLT[c] in PPP [t]**	**PLT[t] in PPP [c]**	**PLT[t] in PPP [t]**
1	43.9	11.8	40.8	16.2
2	49.2	45.3	35.4	34.2
3	34.6	12.4	27.3	14.7
4	39.7	27.7	59.0	43.8
5	48.9	21.3	38.3	29.2
Mean	43.3	23.7	40.2	27.6
Sd	6.2	13.8	11.7	12.3

PLT[c] - washed control platelets; PLT[t] - washed treated platelets.
PPP[c] - control plasma; PPP[t] - treated plasma.

$P < 0.02$, when A compared with B.
$P < 0.05$, when C compared with D.
NS, when A compared with C, and when B compared with D

3-1. Clinical Haematological Parameters

HAEMOGLOBIN (Normal range : F 11.5-16.5 g / dL; M 13.0-18.0 g / dL)

HOU-treated group

Subject No		Pre-treatment	Post-treatment
1	F	12.2	12.7
2	F	13.7	12.8
3	M	15.4	14.8
4	M	15.2	13.5
5	F	14.6	14.4
6	M	16.2	15.9
7	M	13.8	14.3
8	M	14.4	13.9
9	M	14.8	14.2
10	F	13.0	12.3
11	M	14.6	15.2
12	M	15.9	13.9
13	M	13.5	14.1
14	M	14.6	13.9
Mean		14.42	13.99
Sd		1.10	0.97

Control group

Subject No		Pre-treatment	Post-treatment
1	M	14.0	13.6
2	M	15.3	14.9
3	M	14.4	14.6
4	M	15.5	14.7
5	M	14.8	14.1
Mean		14.80	14.38
Sd		0.62	0.52

WHITE BLOOD CELLS (Normal range : 4.0-12.0 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	7.2	8.3
2	8.5	9.9
3	9.8	6.8
4	5.4	6.0
5	5.1	5.7
6	5.0	5.3
7	5.0	5.9
8	7.5	7.5
9	5.3	5.4
10	7.7	10.1
11	4.3	5.3
12	4.7	5.6
13	5.6	6.5
14	5.1	4.8
Mean	6.15	6.65
Sd	1.66	1.70

Control group

Subject No	Pre-treatment	Post-treatment
1	5.5	6.0
2	9.3	7.8
3	6.3	9.2
4	6.5	6.7
5	6.4	6.7
Mean	6.80	7.28
Sd	1.45	1.25

PLATELET (Normal range : 150-400 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	287	325
2	284	253
3	206	201
4	263	259
5	324	276
6	251	251
7	266	262
8	359	316
9	205	207
10	264	275
11	219	231
12	229	205
13	204	223
14	263	246
Mean	258.85	252.14
Sd	45.61	38.06

Control group

Subject No	Pre-treatment	Post-treatment
1	233	225
2	274	254
3	317	317
4	277	294
5	256	216
Mean	271.40	261.20
Sd	30.94	43.57

Hct (Normal range : F 0.37-0.47 L / L; M 0.49-0.54 L / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1 F	0.350	0.355
2 F	0.398	0.365
3 M	0.447	0.426
4 M	0.432	0.397
5 F	0.413	0.424
6 M	0.466	0.461
7 M	0.400	0.409
8 M	0.436	0.406
9 M	0.434	0.415
10 F	0.377	0.359
11 M	0.407	0.432
12 M	0.463	0.410
13 M	0.398	0.419
14 M	0.424	0.410
Mean	0.417	0.406
Sd	0.032	0.029

Control group

Subject No	Pre-treatment	Post-treatment
1 M	0.416	0.390
2 M	0.443	0.418
3 M	0.417	0.409
4 M	0.443	0.431
5 M	0.429	0.404
Mean	0.429	0.410
Sd	0.013	0.015

RED BLOOD CELLS (Normal range : F 3.8-5.8 x 10^{12} / L; M 4.5-6.5 x10^{12} / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1 F	3.95	3.98
2 F	4.76	4.38
3 M	5.12	4.90
4 M	4.72	4.31
5 F	4.66	4.76
6 M	5.31	5.27
7 M	4.31	4.34
8 M	5.08	4.70
9 M	5.10	4.96
10 F	4.45	4.27
11 M	4.57	4.79
12 M	5.49	4.89
13 M	4.39	4.59
14 M	5.02	4.87
Mean	4.78	4.64
Sd	0.42	0.34

Control group

Subject No	Pre-treatment	Post-treatment
1 M	4.52	4.16
2 M	4.74	4.41
3 M	4.96	4.62
4 M	4.88	4.73
5 M	4.80	4.51
Mean	4.78	4.48
Sd	0.16	0.21

MCV (Normal range : 78-98 fl)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	88.5	89.2
2	83.6	83.2
3	87.2	87.0
4	91.6	91.9
5	88.5	89.1
6	87.7	87.4
7	92.8	94.2
8	85.7	86.3
9	85.1	83.7
10	84.8	84.0
11	89.1	90.1
12	84.2	83.8
13	90.6	91.2
14	84.5	84.2
Mean	87.42	87.52
Sd	2.91	3.52

Control group

Subject No	Pre-treatment	Post-treatment
1	92.0	93.6
2	93.4	94.7
3	88.8	88.5
4	90.7	91.1
5	89.3	89.4
Mean	90.84	91.46
Sd	1.90	2.65

MCH (Normal range : 27-32 pg)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	30.9	32.0
2	28.7	29.2
3	30.1	30.3
4	32.1	31.2
5	31.4	30.2
6	30.5	30.1
7	32.1	33.0
8	28.3	29.6
9	29.0	28.7
10	29.3	28.8
11	31.9	31.7
12	29.0	28.4
13	30.8	30.7
14	29.1	28.6
Mean	30.22	30.17
Sd	1.33	1.41

Control group

Subject No	Pre-treatment	Post-treatment
1	31.1	32.6
2	32.2	33.8
3	30.8	31.6
4	31.8	31.0
5	30.8	31.3
Mean	31.34	32.06
Sd	0.63	1.14

RDW (Normal range : 11.0-16.0)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	12.7	12.9
2	12.3	12.4
3	11.7	12.1
4	12.2	11.8
5	12.2	12.1
6	12.0	11.9
7	12.3	12.6
8	12.2	12.5
9	12.3	12.3
10	12.6	12.6
11	12.0	11.7
12	12.0	12.2
13	12.6	12.8
14	12.7	12.6
Mean	12.27	12.32
Sd	0.29	0.37

Control group

Subject No	Pre-treatment	Post-treatment
1	12.7	12.8
2	12.1	12.1
3	12.7	12.4
4	12.1	12.4
5	12.3	12.6
Mean	12.38	12.46
Sd	0.30	0.26

NEUTROPHILS (Normal range : 2.0-7.5 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	4.4	5.2
2	5.8	7.2
3	7.2	4.2
4	2.8	3.0
5	1.8	2.2
6	2.1	2.3
7	3.1	3.5
8	3.8	4.2
9	3.1	3.3
10	4.6	6.8
11	1.8	2.6
12	2.9	3.2
13	2.5	4.2
14	2.0	1.8
Mean	3.42	3.83
Sd	1.59	1.63

Control group

Subject No	Pre-treatment	Post-treatment
1	2.3	3.1
2	5.7	4.3
3	3.5	5.8
4	3.5	3.6
5	3.2	3.7
Mean	3.64	4.10
Sd	1.25	1.04

EOSINOPHILS (Normal range : 0.1-0.4 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	0.1	0.1
2	0.1	0.1
3	0.1	0.1
4	0.5	0.4
5	0.3	0.3
6	0.3	0.4
7	0.1	0.1
8	0.4	0.4
9	0.2	0.1
10	0.2	0.1
11	0.1	0.2
12	0.4	0.4
13	0.2	0.2
14	0.0	0.0
Mean	0.21	0.20
Sd	0.14	0.14

Control group

Subject No	Pre-treatment	Post-treatment
1	0.1	0.1
2	0.6	0.4
3	0.2	0.3
4	0.3	0.3
5	0.3	0.5
Mean	0.30	0.32
Sd	0.18	0.14

BASOPHILS (Normal range : 0 - 0.1 x 10^9 /L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	0.0	0.0
2	0.0	ND
3	0.0	ND
4	0.0	0.0
5	0.1	0.0
6	0.0	0.1
7	0.1	ND
8	ND	0.0
9	0.1	ND
10	0.0	ND
11	0.1	0.1
12	0.0	0.0
13	0.0	ND
14	0.1	0.0
Mean	0.04	0.03
Sd	0.05	0.05

Control group

Subject No	Pre-treatment	Post-treatment
1	0.0	0.2
2	0.1	0.1
3	0.0	0.1
4	0.0	0.0
5	0.0	0.0
Mean	0.02	0.08
Sd	0.04	0.08

LYMPHOCYTES (Normal range : 1.5 - 4.0 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	2.1	2.4
2	2.0	2.6
3	2.0	2.4
4	1.5	2.0
5	2.5	2.5
6	1.8	1.8
7	1.4	1.8
8	2.0	2.2
9	1.3	1.4
10	2.5	2.8
11	1.6	1.7
12	0.9	1.3
13	2.3	2.0
14	2.5	2.6
Mean	1.88	2.10
Sd	0.49	0.46

Control group

Subject No	Pre-treatment	Post-treatment
1	2.3	2.1
2	2.8	2.4
3	2.0	2.4
4	2.2	2.4
5	2.0	1.7
Mean	2.26	2.20
Sd	0.32	0.30

MONOCYTES (Normal range : 0.2 - 0.8 x 10^9 / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	0.6	0.6
2	0.6	ND
3	0.5	0.1
4	0.6	0.6
5	0.5	0.7
6	0.8	0.7
7	0.4	0.5
8	1.4	0.7
9	0.6	0.6
10	0.4	0.4
11	0.6	0.7
12	0.5	0.7
13	0.6	0.1
14	0.5	0.4
Mean	0.61	0.52
Sd	0.24	0.21

Control group

Subject No	Pre-treatment	Post-treatment
1	0.8	0.5
2	0.2	0.6
3	0.6	0.6
4	0.5	0.4
5	0.9	0.8
Mean	0.6	0.58
Sd	0.27	0.14

3-2. Clinical Biochemistry Parameters

UREA (Normal range : 2.5-6.5 mmol / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	4.8	5.4
2	3.7	5.4
3	2.8	5.0
4	5.0	5.9
5	4.7	3.7
6	4.5	3.5
7	3.4	5.3
8	4.5	4.6
9	5.0	5.8
10	3.3	3.6
11	4.6	3.0
12	3.2	5.2
13	7.5	5.4
14	5.9	4.0
Mean	4.49	4.70
Sd	1.22	0.95

Control group

Subject No	Pre-treatment	Post-treatment
1	6.0	4.2
2	5.9	5.5
3	4.6	4.0
4	3.6	3.9
5	3.9	4.0
Mean	4.80	4.32
Sd	1.11	0.66

SODIUM (Normal range : 135-145 mmol / L)

HOU-treated group

Subject No	Pre-treatmen	Post-treatment
1	140	142
2	139	141
3	141	144
4	141	143
5	141	142
6	139	143
7	141	142
8	139	142
9	140	141
10	141	139
11	141	142
12	141	140
13	143	142
14	143	142
Mean	140.71	141.78
Sd	1.26	1.25

Control group

Subject No	Pre-treatmen	Post-treatment
1	140	140
2	140	141
3	139	141
4	142	145
5	140	146
Mean	140.20	142.60
Sd	1.09	2.70

POTASSIUM (Normal range : 3.5-5.0 mmol / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	4.6	4.5
2	4.0	4.2
3	3.9	4.2
4	4.3	4.4
5	4.0	3.5
6	4.2	4.2
7	4.0	5.0
8	3.9	4.5
9	4.1	4.5
10	3.9	4.3
11	4.4	3.8
12	4.1	3.9
13	4.0	4.0
14	4.0	3.9
Mean	4.10	4.20
Sd	0.20	0.37

Control group

Subject No	Pre-treatment	Post-treatment
1	3.6	4.3
2	4.2	4.6
3	4.2	4.4
4	4.4	4.1
5	4.2	4.5
Mean	4.12	4.38
Sd	0.30	0.19

BICARBONATE (Normal range : 22-32 mmol / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	32	34
2	27	28
3	30	30
4	34	31
5	25	29
6	31	30
7	26	37
8	25	37
9	28	28
10	26	26
11	29	31
12	36	32
13	32	30
14	27	30
Mean	29.14	30.92
Sd	3.46	3.19

Control group

Subject No	Pre-treatment	Post-treatment
1	27	36
2	25	36
3	28	29
4	34	29
5	31	32
Mean	29.00	32.40
Sd	3.53	3.50

CREATININE (Normal range : 45-120 μmol / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	79	90
2	93	89
3	100	101
4	92	99
5	ND	ND
6	75	85
7	ND	96
8	ND	109
9	ND	ND
10	ND	ND
11	ND	ND
12	88	93
13	87	ND
14	ND	ND
Mean	87.71	95.25
Sd	8.51	7.68

Control group

Subject No	Pre-treatment	Post-treatment
1	ND	ND
2	ND	115
3	84	88
4	91	96
5	81	81
Mean	85.33	95.00
Sd	5.13	14.67

TOTAL PROTEIN (Normal range : 60-88 g / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	68	73
2	77	74
3	72	69
4	75	68
5	78	77
6	71	72
7	69	75
8	76	72
9	77	76
10	81	73
11	71	75
12	80	73
13	68	ND
14	82	77
Mean	74.64	73.38
Sd	4.81	2.75

Control group

Subject No	Pre-treatment	Post-treatment
1	72	70
2	69	67
3	77	74
4	70	70
5	75	74
Mean	72.60	71.00
Sd	3.36	3.00

ALBUMIN (Normal range : 35-50 g / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	43	46
2	47	44
3	46	44
4	49	46
5	47	46
6	49	48
7	44	51
8	47	47
9	46	46
10	48	44
11	47	51
12	51	47
13	44	NB
14	49	47
Mean	46.92	46.69
Sd	2.23	2.28

Control group

Subject No	Pre-treatment	Post-treatment
1	46	47
2	45	45
3	50	48
4	46	46
5	47	46
Mean	46.80	46.40
Sd	1.92	1.14

BILIRUBIN (Normal range : 3-21 g / L)

HOU-treated group

Subject No	Pre-treatment	Post- treatment
1	9	12
2	11	9
3	9	7
4	25	18
5	11	12
6	22	32
7	10	8
8	10	3
9	20	20
10	10	6
11	14	8
12	11	13
13	7	ND
14	8	16
Mean	12.64	12.61
Sd	5.58	7.61

Control group

Subject No	Pre-treatment	Post- treatment
1	16	13
2	17	6
3	4	2
4	13	10
5	9	10
Mean	11.80	8.20
Sd	5.35	4.26

ALK.PHOS (Normal range : 80-300 u / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	144	173
2	185	163
3	165	148
4	146	140
5	203	205
6	260	245
7	109	108
8	137	132
9	223	236
10	229	227
11	130	130
12	190	137
13	289	NB
14	172	169
Mean	184.42	170.23
Sd	51.91	44.60

Control Group

Subject No	Pre-treatment	Post-treatment
1	128	122
2	158	145
3	162	153
4	143	141
5	218	194
Mean	161.80	151.00
Sd	34.16	26.59

GAMMA G.T.(Normal range : 7-50 u / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	8	8
2	17	19
3	21	15
4	13	13
5	9	10
6	12	10
7	8	8
8	9	9
9	8	9
10	9	10
11	13	15
12	13	13
13	41	ND
14	29	34
Mean	15.00	13.30
Sd	9.55	7.02

Control group

Subject No	Pre-treatment	Post-treatment
1	11	10
2	22	21
3	57	50
4	9	10
5	20	17
Mean	23.80	21.60
Sd	19.38	16.56

AST (Normal range : 0-40 u / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	14	18
2	20	19
3	20	18
4	19	16
5	20	18
6	23	18
7	15	18
8	12	12
9	19	19
10	15	13
11	21	12
12	18	17
13	32	ND
14	20	29
Mean	19.14	17.46
Sd	4.80	4.29

Control group

Subject No	Pre-treatment	Post-treatment
1	16	15
2	20	19
3	15	14
4	12	14
5	20	19
Mean	16.60	16.20
Sd	3.43	2.58

LD (Normal range : 230-460 u / L)

HOU-treated group

Subject No	Pre-treatment	Post-treatment
1	285	290
2	263	282
3	323	261
4	277	295
5	253	234
6	365	291
7	269	270
8	244	237
9	315	353
10	249	238
11	280	237
12	334	309
13	273	NB
14	271	343
Mean	285.78	280.00
Sd	35.41	39.39

Control group

Subject No	Pre-treatment	Post-treatment
1	276	247
2	318	264
3	232	222
4	230	271
5	357	334
Mean	282.60	267.60
Sd	55.13	41.64

4-1. Isolation of mononuclear cells from peripheral blood

In general, methods for the isolation of mononuclear cells from circulating blood and bone marrow have employed mixtures of polysaccharide and radiopaque contrast medium (Boyum, 1968). Histopaque 1077 from Sigma is a solution of polysucrose and sodium diatrizoate, with a density of 1.077. This medium facilitates rapid recovery of viable mononuclear cells from small volumes of blood. This procedure is suitable for studying cell -mediated lympholysis and for human lymphocyte antigen (HLA) typing.

(1) 3.0 ml HISTOPAQUE-1077 are carefully layered over 3.0 mL anticoagulated venous blood in a 15 mL centrifuge tube.

(2) Centrifuge at 400 xg for exactly 30 minutes at room temperature. Centrifugation at lower temperatures, such as 4°C may result in cell clumping and poor recovery. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and sediment; whereas, lymphocytes and other mononuclear cells remain at the plasma - HISTOPAQUE-1077 interface. Erythrocyte contamination is negligible.

(3) After centrifugation, carefully aspirate, with a Pasteur pipet, the upper layer to within 0.5 cm of the opaque interface containing mononuclear cells. Discard upper layer.

(4) Carefully transfer the opaque interface, with a Pasteur pipette, into a clean centrifuge tube.

(5) The cell pellet are washed 3 times with DMEM; centrifuge at 250 xg for 10 minutes. Most extraneous platelets are removed by low speed centrifugation duing the washing step. The cells are resuspended in DMEM. (see diagram below).

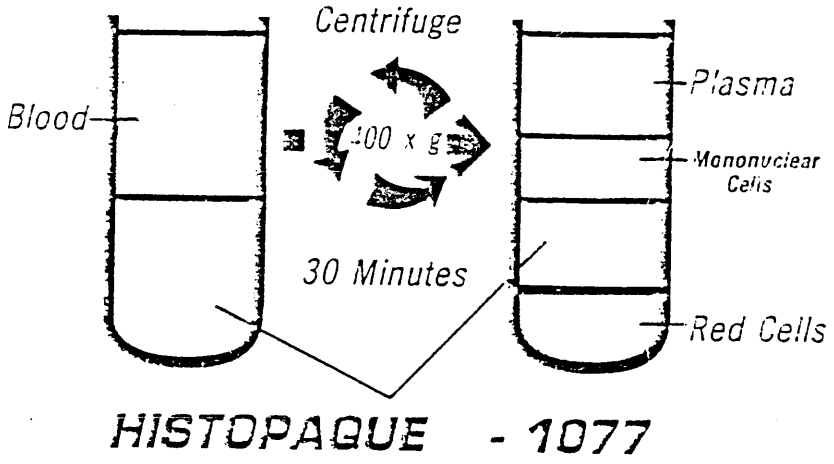


Diagram of the isolation of mononuclear cells from peripheral blood

4-2. The alkaline phosphatase / anti-alkaline phosphatase (APAAP) method

4-2-1. Principle of the APAAP

The alkaline phosphatase / anti-alkaline phosphatase (APAAP) method utilizes three reagents: Primary and secondary antibodies, and APAAP enzyme immune complex - comprising the alkaline phosphatase and an antibody against alkaline phosphatase. The primary antibody is specific for the antigen on the cells. The secondary or "link" antibody is capable of binding to both the primary and to the APAAP enzyme immune complex, because the primary antibody and the antibody against alkaline phosphatase in the APAAP enzyme immune complex are produced in the same animal species. The alkaline phosphatase enzyme is visualized via a substrate-chromagen reaction (see diagram below). The activity of the endogenous alkaline phosphatase is effectively inhibited by the use of levamisole during the colour development reaction (Erber, et al, 1984; Ponder, et al, 1984; Mason, 1985; Cordell, et al, 1984). Levamisole does not denature sensitive surface antigens.

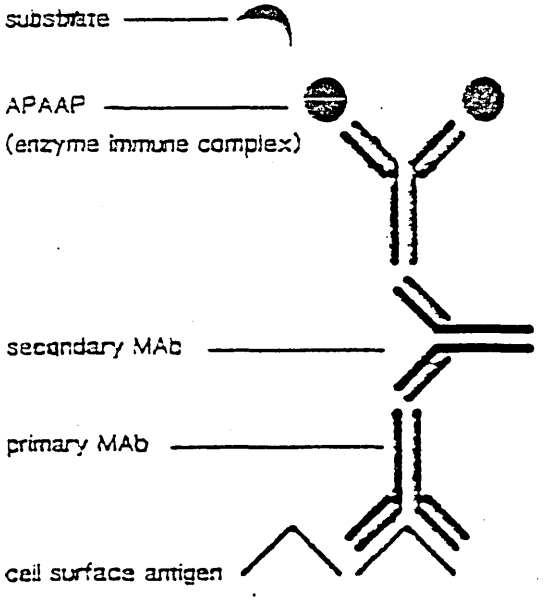


Diagram of the principle of the APAAP

4-2-2. Procedure of the APAAP

The monoclonal alkaline phosphatase / anti-alkaline phosphatase (APAAP) staining procedure was adapted from the method of Mason (Mason, 1985).

The use of phosphate buffers is not recommonded since this may compete with phosphates in the substrate. Before fixation, stored slides were allowed to reach room temperature and were then unwrapped. Slides were fixed in acetone: methanol : formalin for 90 seconds. The excess fixative was shaken off and the slides were then immediately washed in Tris buffered saline for 5 minutes.

Staining step:

(1) Apply primary monoclonal antibody (see Table 4-1) - Optimum concentration of mouse primary antibody is applied and incubated in a moist chamber for 30 minutes at room temperature. Negative controls were added TBS by omission of this primary mAb stage.

(2) Slides were washed 1-2 minutes, to remove traces of unbound antibody. Draining and bloting slides.

(3) Apply secondary antibody (Rabbit anti-mouse immunoglobulins DAKOPATTS Code No. Z 259 at 1 / 25 dilution) - Incubate with a secondary antibody in a moist chamber for 30 minutes at room temperature.

(4) Wash in TBS for 1-2 minutes. Draining and bloting slides.

(5) Apply APAAP complex (DAKOPATTS Code No. D 651 at 1 / 50 dilution) and incubate in a moist chamber for 1 hour at room temperature.

(6) Wash for 1-2 minutes in TBS.

The intensity of final staining can be greatly enhanced at this point by repeating steps 3-6. When carriying out this cycle, incubation times were reduced to 10 minutes.

(7) Add filtered alkaline phosphatase substrate contaning levamisole and incubate for 15 minutes at room temperature, then wash the slides in TBS and tap water. Levamisole is used to inhibit endogenous enzyme activity.

(8) Counterstain - 3-5 minutes with Mayer's Hematoxylin. Rinse gently in running tap water. Rinse in distilled water.

(9) Mount slides with Mounting medium (Glycergel from DAKO).

APAAP staining reagents:

Fixatives Acetone : methanol : formalin = 10 : 10 : 1

Buffer Tris buffered saline (TBS)
0.05 M Tris
0.15 M NaCl
pH 7.6 adjusted using 1 N HCl

Substrate Naphthol AS-MX phosphate, free acid 2 mg

Dimethylformamide 0.2 ml

0.1 M TRIS buffer pH 8.2 9.8 ml

1 M Levamisole 10 μl

Fast-Red TR salt 10 mg

Prepare this solution dissolving the Naphthol AS-MX phosphate in dimethylformamide in a glass tube. Dilute to 10 ml with the Tris buffer pH 8.2. Add levamisole to block endogenous alkaline phosphatase activity. This solution can be made up in 500 μl aliquots and stored at - 20°C. Immediately before staining, dissolve the Fast-Red salt in the substrate solution and filter directly onto the slides.

Staining by the APAAP method utilising Fast Red chromogen causes formation from bright red to vivid red precipitate (depending on the amount of antigen present) around the target cell membrane, whereas nuclei and negative cells remained blue only when a hematoxylin counterstained was used.

4-3. The principle of EIA for 6-keto-prostaglandin $F_{1\alpha}$

The assay operates on the principle of competition between a horseradish peroxidase (HRP) labelled 6-keto-prostaglandin F1a conjugate and the 6-keto-prostaglandin F1a in the sample for a limited number of binding sites on the anti-6-keto-prostaglandin F1a rabbit antibody precoated microtitre plates (see diagram below). This provides a rapid and sensitive method for the determination of 6-keto-prostaglandin F1a at a concentration range of 5 to 400 pg / ml.

1) One Step Incubation

The sample or standard solution is first added to the primary antibody precoated microtiter plate. Next, the diluted antigen-enzyme conjugate is added and the plate is shaken and incubated at room temperature for one hour. During the incubation, the competition for binding sites takes place.

2) Washing

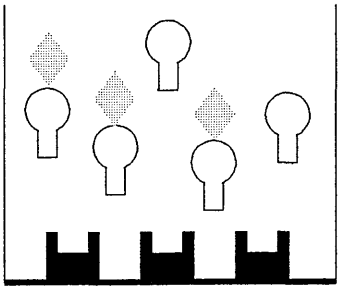
The plate is then washed, to remove all the unbound reagents.

3) Development

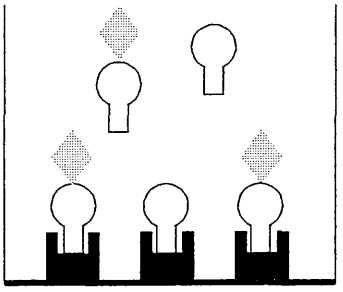
The bound antigen-enzyme conjugate is detected by the addition of enzyme substrate; 3,3'-5,5' Tetramethylbenzidine (TMB) plus hydrogen peroxide (H_2O_2), which generate an optimal blue color after 30 minutes.

4) Data Collection

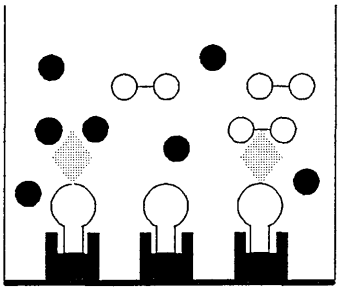
Absorbance is measured at 650nm with a microtiter plate reader, and the standard curve is used to determine the concentration of the unknown samples. The extent of colour development is inversely proportional to the amount of 6-keto-PGF_{1a} in the sample or standard. The sensitivity of the assay can be enhanced and terminated by the addition of 1 N H_2SO_4 which converts the blue product to an intense yellow colour with an absorbance maximum at 450 nm.



(1) One Step Incubation

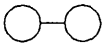


(2) Washing



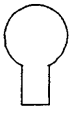
(3) Development

Immobilized antibody

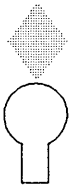


Subtrate

Coloured product



Antigen to be assayed
(ANALYTE)



ANTIGEN-ENZYME
(CONJUGATE)

Diagram of assay principle of EIA for 6-keto $PGF_{1\alpha}$

4-4. Reagents, equipment and suppliers

4-4-1 Reagents

All reagents unless otherwise indicated were obtained from Sigma Chemical Co, Poole, Dorset, UK.

APAAP complex	DAKOPATTS, Denmark, code No. D651.
1 mm diameter cadmium wire	Aldrich Chemical Company, Inc. USA.
IFN-gamma ELISA kit	Endogen Inc, 451 D Street, Boston, MA, USA.
6-keto-$PGF_{1\alpha}$ ELISA kit	Cascade Biochem Ltd, The Innovation Centre, University Campus Reading, Berkshire, England.
6-keto-$PGF_{1\alpha}$ RIA kit	Amersham International plc, UK.
PDGF RIA kit	Amersham International plc, UK.
IL-2 test kit	T Cell Diagnostics, Inc. 38 Sidney Street, Cambridge, MA 02139

4-4-2 Equipment

Aggregometer	ADG Instruments Ltd, Codicote, Herts, UK
Aggregometer cuvette	ADG Instruments Ltd, Codicote, Herts, UK
Amprep C2 100 mg minicolumn	Amersham International plc, UK.
Centrifuges	MSE MISTRAL 2L.
Chart recorder	Talbot Instruments, Alderley Edge, Cheshire, UK.
Coulter	Coulter Model S. Plus, Hialeah, Florida, USA.

Disposable sterile pack for HOU-treatment	
	Quarzlampenfabrik, Dr Muller, Gmbh,
	Essen, Germany.
Filter Papers	Whatman, Maidstone, Kent, UK.
Gamma counter	LKB.
MAXI SCREEN Combi 6A test strip	
	Cambrige Selfcare Diagnostics limited, UK.
Microcentrifuge	MSE Micro Centaur SANYO.
Microtiter plate reader	Labsystems Multiskan MCC/340.
Multispot slide	CA Hendley, Essex Ltd, Oakwood Hill Industrial
	Estate, Loughton, Essex, England.
Ozon-O-Med	Quarzlampenfabrik, Dr Muller, Gmbh, Essen,
	Germany.
Ozone monitor	Humares, Karlsruhe, Germany.
Pipettes	Eppendorf
Pump	302S, WATSON-MARLOW Limited, England.
Spectrophotometer	Ultraspec 4050 LKB
Stirrer bar	Aggregometer Model 1002, ADG Instruments
	Ltd, Codicote, Herts, UK.
Visbing tube	Fisons, Leicester, UK
Vorter Mixer	MT20, Chiltern
Water Bath	Grant Cambrige

4-4-3 Reagents of platelet aggregation

ADP Adenosine 5'-diphosphate, sodium salt Grade III from yeast ATP, anhydrous. Frozen solutions at pH 6.8 are more stable. A 10 mmol / L stock solution in saline was prepared and stored in 0.5 ml aliquots at -20°C. Solutions of ADP for platelet aggregation testing were prepared at 5.0 mmol / L, 1.0 mmol / L, 0.5 mmol / L, 0.05 mmol / L and 0.01 mmol / L concentrations in saline (Table 4-4-3).

Collagen Type III, acid solution, from calf skin. A 10 mg / ml stock solution was prepared and stored at 4°C for 2 weeks. For use, it was diluted in the PBS to obtain 1mg / ml concentration (Table 4-4-3).

Thrombin from bovine plasma. A 50 u /ml stock solution in saline was prepared and stored at -20°C. For use, it was diluted in saline to obtain the concentrations of 1u / ml and 5 u /ml (Table 4-4-3).

Calcium ionophone A 23187 $Ca^{++} + Mg^{++}$salt, 500 μg / ml in DMSO (dimethylsulphoxide) at -20°C, diluted in saline to give 10 μg /ml, 50 μg /ml and 100 μg /ml solutions (Table 4-4-3).

Adrenaline 1-Epinephrine bitartarate, 1 mmol / l stock solution in saline. It was stored at -20°C in 0.5 ml volumes. For use, an 0.1 mmol /l solution in saline (Table 4-4-3) was prepared.

Table 4-4-3. Reagents for the study of platelet aggregation

Aggregating agent	Stock solution		Working solutions		Final concentration, when 1 volume is added to 9 volumes of PRP
	Diluent	Concentration	Diluent	Concentration	
ADP	Saline	10 mmol / L	Saline	5 mmol / L	0.5 mmol / L
				1 mmol / L	0.1 mmol / L
				0.5 mmol / L	0.05 mmol / L
				0.1 mmol / L	0.01 mmol / L
				0.05 mmol / L	0.005 mmol / L
				0.01 mmol / L	0.001 mmol / L
Collagen	PBS	10 mg /ml	PBS	1 mg /ml	0.1 mg /ml
Thrombin	Saline	50 u / ml	Saline	5 u / ml	0.5 u / ml
				1 u / ml	0.1 u / ml
Calcium-Ionophone-A 23187	DMSO	500 μg / ml	Saline	100 μg / ml	10 μg /ml
				50 μg /ml	5 μg /ml
				10 μg /ml	1 μg /ml
Adrenaline	Saline	1 mmol / L	Saline	0.1 mmol / L	0.01 mmol / L